EPOXIDATION BY AN ENZYME SYSTEM OF *PSEUDOMONAS OLEOVORANS*

A THESIS

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SYSTEM OF PSEUDOMONAS OLEOVORANS

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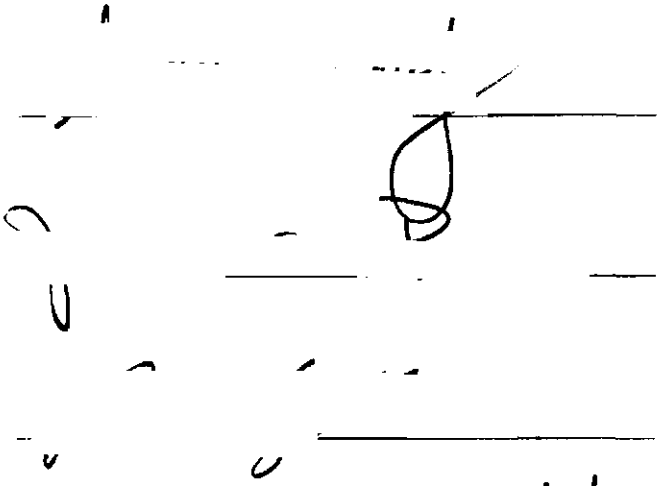

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SUMMARY

A study of the epoxidation reaction by an enzyme system of P. oleovorans was carried out from a mechanistic, stereochemical and a synthetic viewpoint. In previous work, it has been shown that this enzyme system catalyzes the conversion of terminal olefins to their corresponding 1,2-oxides with a high degree of stereoselectivity and with specificity patterns far different from those expected on the basis of chemical reactivity in nonenzymatic epoxidation reactions. In order to probe directly the mechanism of oxygen insertion in this system, the substrate trans,trans-1,8-dideuterio-1,7-octadiene was synthesized and used as a substrate in the enzymatic reaction. The 7,8-epoxy-1-octene product was isolated and its structure analyzed using partially relaxed proton Fourier Transform NMR spectroscopy. While the equilibrium NMR spectrum of the epoxide product exhibited serious overlapping of signals due to the presence of fully protonated and both cis and trans deuterated species, use of the partially relaxed FT NMR technique allowed isolation of the spectra of the various species, quantitative analysis of the product mixture, and a relaxation time analysis of both the fully protonated and deuterated species which helped confirm the spectral

assignments. On the basis of these analyses, it is concluded that the enzymatic reaction proceeds primarily with inversion of the original olefin geometry. In contrast, a similar analysis on the product obtained via peracid epoxidation of the trans deuterated olefin revealed the presence of only the trans deuterated epoxide. Control experiments indicate that no isomerization of either the epoxide or olefin functionalities occurs under the reaction conditions. These results are consistent with the conclusion that a simple "oxenoid" mechanism is not operative in this enzymatic reaction. These results, when coupled with the finding that 90% of the 7,8-epoxy-1-octene produced from octadiene is of the R(+) configuration, provide a basis by which several mechanistic proposals were evaluated.

An investigation of the stereochemistry of the enzymatically produced 1,2-7,8-diepoxyoctane by ORD and NMR analysis using a chiral shift reagent revealed that production of diepoxides from simple olefins by this enzyme system proceeds with a high degree of stereoselectivity. Furthermore, from an analysis of the diepoxide produced from racemic 7,8-epoxy-1-octene, it is clear that the presence of a preformed asymmetric center at one end of the substrate profoundly affects the stereochemical consequences of enzymatic oxygen insertion into a double bond at the other end of the

molecule. A scheme consistent with our results is that the S-monoepoxide produces predominantly the S,S-diepoxide with the same degree of stereoselectivity observed in the production of R,R-diepoxide from R-monoepoxide but that the R(+) enantiomer reacts more readily.

In our attempt to determine whether the stereoselectivity of the epoxidation reaction is affected by the presence of allylic hydroxyl groups, we found that allylic alcohols are not epoxidated but instead are surprisingly converted to saturated ketones by P. oleovorans. An investigation of the ketonization of 1-octen-3-ol and 2-methyl-1-hepten-3-ol revealed that only the reductase component of P. oleovorans and NADH are required for these reactions. In addition to the allylic alcohols, saturated alcohols and α,β-unsaturated ketones are also converted to their corresponding saturated ketones. Our results establish that these reactions are enzymatic processes, apparently specific for reductase.

Because of the highly unusual features of P. oleovorans, it is clear that this system has considerable potential for synthetic applications. In order to enhance the attractiveness of this system, we developed simple procedures using either whole cells or cell free extracts by which optically active epoxides can be produced on a preparative scale. These procedures also provide for comparable yields in the syntheses of saturated ketones

from allylic alcohols, although an optimization study for these reactions were not carried out.

CHAPTER I

INTRODUCTION

Much of the current interest in biological oxidations concerns the involvement of molecular oxygen as a substrate. In 1955, Hayaishi, et.al. used a heavy oxygen isotope as a tracer in $^{18}O_2$ and $H_2{}^{18}O$ and demonstrated that both atoms of an oxygen molecule were incorporated into an organic compound in an enzymatically catalyzed reaction.[1] Concurrently, Mason et.al.[2] similarly demonstrated the enzymatically catalyzed incorporation of one atom of an oxygen molecule into the substrate with the other oxygen atom being reduced to H_2O. Since then, the direct incorporation of molecular oxygen into organic compounds in living systems has been well established. These enzymes which catalyze the activation of molecular oxygen and the subsequent incorporation of one or both atoms of the oxygen molecule into the substrate are termed oxygenases. The terms "mono" and 'di' oxygenases are assigned respectively to the enzymes catalyzing these two types of reactions. These differ from the oxidases which reduce the oxygen molecule either to hydrogen peroxide or to two molecules of water without the incorporation of molecular oxygen into substrate. The

mono-oxygenases are sometimes referred to as mixed function oxidases since they carry out both oxidase and oxygenase activity.

Oxygenases, which are widely distributed in nature,[3] represent one of the most significant of all enzymes in that they catalyze key steps in the biosynthesis and degradation of many metabolites ranging from amino acids and lipids to vitamins and hormones. These enzymes catalyze the hydroxylation of both aromatic and aliphatic compounds, the epoxidation of olefins as well as the cleavage of aromatic double bonds, dealkylation, decarboxylation, and deamination reactions.

In organic chemistry, such oxygen fixation reactions have essentially been confined to the involvement of either high energy species or transition metal catalysts in order to overcome the high energy barrier associated with the combination of a ground state triplet oxygen molecule to a ground state singlet organic compound. Oxygenases overcome this difficulty without the involvement of high energy species. Although some oxygenases contain transition metal ions, others contain organic cofactors. Thus, the mechanism by which molecular oxygen is activated and subsequently incorporated into organic compounds represents one of the most intriguing and challenging problems in biological chemistry today.

The subject of this dissertation is an oxygenase system isolated from the bacterium Pseudomonas oleovorans. This enzyme system, consisting of three protein components, was shown by Coon and coworkers[4-12] to catalyze the terminal hydroxylation of alkanes and fatty acids in the presence of NADH and molecular oxygen. The three protein components have been identified as rubredoxin, a reductase, and ω-hydroxylase. Rubredoxin is an ($FeCyS_4$) iron-sulfur protein, containing two single iron active sites in a polypeptide of molecular weight 19,000. This protein which has been purified to homogeneity and its amino acid sequence determined, apparently serves as an electron carrier in the system. Reductase is a flavoprotein of molecular weight 55,000, containing one flavin adenine dinucleotide per molecule. This protein has been purified to homogeneity and serves to transfer electrons from NADH to rubredoxin. ω-Hydroxylase is a protein of very high molecular weight (10^6) and recent studies indicate that it contains a non heme iron and consists of subunits of molecular weight 42,000.

A feature of this mono-oxygenase system which sets it apart from other well-known hydroxylation systems is the nature of the prosthetic group. In the well-characterized three component camphor hydroxylase system of Pseudomonas putida, cytochrome P-450 serves as the functional oxygenase component.[13-15] Cytochrome P-450 has also been established

to be functional in the octane oxidations by extracts of Corynebacterium,[16] in the steroid 11-β-hydroxylase system from adrenocortical mitochondria,[17,18] and in the oxidations of steroids and polycyclic hydrocarbons by liver microsomes.[19,20] In contrast, the ω-hydroxylase system of P. oleovorans does not contain cytochrome P-450 but apparently requires a non heme iron for activity.

A second feature which distinguishes the P. oleovorans system from other hydroxylation systems is the nature of the electron transferring protein. Both adrenodoxin of adrenocortical mitochondria and putidaredoxin of the P. putida system are non heme iron proteins of the ferredoxin type ($CyS_4Fe_2S_2$), containing two iron atoms and two labile sulfides.[14] Rubredoxin of the P. oleovorans system does not contain a labile sulfide in the prosthetic group. In addition, neither adrenodoxin nor putidaredoxin can accept electrons from the reductase component of P. oleovorans.[11] On the other hand, rubredoxins from several anaerobic bacteria can accept electrons from reductase but yet cannot substitute for P. oleovorans rubredoxin in the hydroxylation reaction. These unusual features suggest that rubredoxin of P. oleovorans may be directly involved in the binding and activation of oxygen.

In addition to the previosuly known hydroxylation reactions, it has recently been shown by May and coworkers[21-30] that the same enzyme system of P. oleovorans

is also capable of catalyzing the conversion of terminal olefins to their corresponding 1,2-oxides. The epoxidation reaction requires the presence of all three protein components as well as NADH and molecular oxygen. A 1:1:1 stoichiometry is obtained between the amount of NADH oxidized, the amount of product formed and the amount of oxygen consumed, indicating that the P. oleovorans system is acting as a mixed function oxidase in catalyzing the epoxidation reaction. 1,7-octadiene which does not contain a hydroxylatible methyl group is converted exclusively to 7,8-epoxy-1-octene and this compound can be further oxidized to give 1,2-7,8-diepoxyoctane. On the other hand, 1-octene which contains both a terminal methyl group and a terminal double bond is converted to both 7-octen-1-ol and 1,2-epoxyoctane with the epoxidation reaction proceeding more readily. The reactivity of the P. oleovorans system towards straight chain olefins is maximal for octadiene and decreases considerably as the chain length is shortened but decreases only slightly as the chain is lengthened. A further interesting and unique feature of the epoxidation reaction is that it proceeds with a high degree of stereoselectivity. 1,7-Octadiene is epoxidated to give preferentially the R isomer of 7,8-epoxy-1-octene and thus represents a true example of an asymmetric synthesis since 1,7-octadiene is a prochiral molecule.

How the stereoselectivity and specificity of this epoxidation reaction of P. oleovorans differs from other bacterial epoxidation systems remains to be established. It has been shown that cells of Pseudomonas aeruginosa grown on saturated hydrocarbons can convert 1-heptene,[31] 1-octene[31,32] and 1-nonene[31] to their corresponding 1,2-oxides. However, the epoxidation is only a minor reaction pathway and the yields of the epoxide products are negligible. The P. oleovorans epoxidation system differs from cytochrome P-450-containing liver microsomes in that several aromatic compounds which are converted to arene oxides by liver microsomes are not substrates for P. oleovorans.

The hydroxylation/epoxidation system of P. oleovorans, like all mono-oxygenase reactions, involves both electron transfer and oxygen insertion steps and there has been considerable speculation concerning the interaction of these events and the nature of the final activated oxygen species. It has been noted that many mono-oxygenase catalyzed reactions show great similarities to those reactions involving carbenes.[33] Carbenes will insert into unactivated alkane carbon-hydrogen bonds to give alkyl derivatives, will add to alkenes to give cyclopropane compounds and will react with aromatic compounds to give norcaradienes and toluenes. Similarly, oxygenases catalyze the hydroxylation of alkanes, the epoxidation of olefins and will convert aromatic compounds to arene oxides and phenols.

Since carbenes are species containing six electrons in the outer valence shell, the great similarities between the reactions of carbenes to those catalyzed by oxygenases suggest an oxygen species with six electrons in the outer valence shell. Hamilton first suggested that an electrophilic "oxenoid" species is generated by these enzymes by a transfer of two electrons prior to or concurrent with the transfer of an oxygen atom to the substrate.[34-37] Among the most well studied of all "oxenoid" reactions in organic chemistry is the peracid epoxidation of olefins and this reaction clearly proceeds through electrophilic attack on the olefin as evidenced for example by the well documented effects of electron donating or electron withdrawing substituents.[38-41] A diagnostic characteristic of this reaction is its absolute syn stereospecificity, even in cases where substituents which could stabilize a carbonium ion intermediate are present.[39-43] Similarly, among the oxo transition metal complexes, which have been considered as possible models for oxygenase reactions,[44] chromyl acetate[45] and molybdenum oxo complexes[46] are known to epoxidize olefins with retention of geometry, and the same is true for transition metal catalyzed epoxidations by alkyl hydroperoxides.[47]

The "oxenoid" mechanism has found particular favor in accounting for cytochrome P-450-containing oxygenases and for the NIH shift which occurs in aromatic hydroxylations.[33,48]

The NIH shift refers to an intramolecular hydrogen migration with concommitant retention of aryl substituents during aromatic hydroxylations and indicates the formation of an arene oxide as an intermediate. The occurrence of the NIH shift in the enzymatic hydroxylation of aromatic compounds has been taken as evidence of an "oxenoid" mechanism. If such a mechanism is operative in these oxygenase catalyzed reactions, it is apparent that the "oxenoid" reagent would not be the same for all these enzymes since various different prosthetic groups (transition metals, hemes, reduced flavins, and reduced pteridines) are required.

To help provide insight into the characteristics of the enzymic reagents, several chemical model systems have been studied. The first hydroxylase model with molecular oxygen was proposed by Udenfriend.[49] The model consisted of an iron II-ethylenediaminetetraacetic acid complex, ascorbic acid and molecular oxygen. This chemical system catalyzes the hydroxylation of aromatic compounds and saturated hydrocarbons as well as the epoxidation of olefins. The Udenfriend system suffers in that it gives a complex mixture of products and the yields of the hydroxylated products are low and it has not been possible to establish the mechanism of these reactions. It does appear, however, that this system does not operate by an "oxenoid" mechanism since this system does not show a significant NIH shift.

A hydroxylating system with new properties, consisting of iron II-2-mercaptobenzoic acid complex with acetone as the solvent was studied by Ullrich.[50,51] In the presence of molecular oxygen, this system hydroxylates aromatic compounds in good yields. However, this system does not show a significant NIH shift and does not epoxidize olefins. Similarly, a chemical system studied by Hamilton and consisting of H_2O_2, Fe^{III} and catechol has also been shown to catalyze aromatic hydroxylations in good yields but without a significant NIH shift.[52] In addition to the metal ion-O_2 model systems, systems composed of reduced flavins and molecular oxygen have been shown to catalyze aromatic hydroxylation reactions. Although product yields are very low, these systems provide chemical models for oxygenases which do not require a transition metal ion but requires instead a reduced flavin.[33,53]

Of all the hydroxylation models currently available, none adequately mimics the enzymatic reaction and considerable speculation remains concerning the mechanism of these chemical systems. Although the oxene mechanism is attractive and reasonable, in no case has an actual enzymic "oxenoid" reagent been identified. There exists a considerable amount of experimental evidence to both support and refute this mechanism.

Thus it appears that the P. oleovorans system provides an excellent choice for mechanistic studies on oxygen

activation. This enzyme system catalyzes both hydroxylation and epoxidation reactions and the alkene epoxidation finds a chemical analogy in peracid epoxidations which are clear examples of an "oxenoid" reaction.

The unusual selectivity of this enzyme system towards terminal double bonds and the high stereospecificity are important not only for mechanistic considerations but also from a synthetic viewpoint. The ability to effect asymmetric syntheses of optically active epoxides from simple olefins with a high degree of optical purity makes this enzyme system a potentially attractive synthetic tool.

Our broad objectives in carrying out the research described in this dissertation were: to probe the mechanism of oxygen insertion into unsaturated hydrocarbons, to further investigate the stereochemical aspects of the epoxidation reaction, and to explore the potential synthetic utility of this enzyme system by developing simple procedures for producing optically active epoxides on a preparative scale and by investigating the possible versatility of this system towards oxidation of other selected organic compounds.

The results of these studies will hopefully provide a better understanding of the biological oxygenation process and its development as a novel catalyst in carrying out the direct incorporation of molecular oxygen into organic compounds.

CHAPTER II

EXPERIMENTAL

Materials

Hydrocarbons, alcohols, ketones and olefins were pruchased from various sources and were of the highest grade commercially available. Inorganic salts were the commercially available reagent grade materials. DNase, RNase, NADH, NAD^+, d,l-α-phosphatidylcholinedipalmitoyl and protocatechuic acid were from Sigma, m-chloroperbenzoic acid, 1M diborane/THF solution and tris [3-(trifluoro-methylhydroxymethylene)-3-camphorato]europium III were purchased from Aldrich. Preswollen DEAE-cellulose (DE-52) was purchased from Whatman, Sepharose from Pharmacia and Biogel A from Biorad. Ultrapure $(NH_4)_2SO_4$ was from Schwarz/Mann.

The organism P. oleovorans strain TF4-1L was originally obtained as a culture stored on a nutrient agar slant and kindly provided by R. D. Schwartz, Exxon Research and Engineering Co., Linden, N. J.

Bacteria and Cell Growth

P. oleovorans strain TF4-1L was used throughout all experiments, and was routinely cultivated in a medium consisting of: $(NH_4)_4HPO_4$, 10g; K_2HPO_4, 5g; Na_2SO_4, 0.5g;

$MgSO_4 \cdot 7H_2O$, 0.4g; $FeSO_4 \cdot 7H_2O$, 0.02g; $MnSO_4 \cdot 4H_2O$, 0.02g; NaCl, 0.02g; H_3BO_3, 0.5g; $CuSO_4 \cdot 5H_2O$, 0.04 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg; $ZnSO_4 \cdot 7H_2O$, 8.0 mg; $CaCl_2$, 50.0 mg; $CoCl_2 \cdot 6H_2O$, 2.0 mg; distilled H_2O to 1 liter.

100 ml of P-1 medium was autoclaved in a 300 ml baffled shake flask for 45 minutes at 121°C, then inoculated with a 5 ml washing of a nutrient agar slant. The solution was supplemented with 1% v/v octane as sole source of carbon and energy. The hydrocarbon had been filter sterilized before addition, using a Millipore filter with a pore size of 1.2 μm. Incubation was carried out at 30°C in a rotary shaker (New Brunswick Scientific, New Brunswick, N. J.) at 290 rpm for 36 hr. at which time an optical density of 5 at 660 nm was obtained and cell growth had entered a stationary phase. The cell solution was centrifuged at 20,000 x g and 4°C for 10 min. after which the reddish brown cell pellets were resuspended in 30 ml medium containing 10% v/v glycerin to give a concentrated sample. Samples were stored in 3 ml volumes in sealed ampoules at -75°C and used as stock cultures for all future preparations. Several microliters of the stock preparation were streaked on a sterile nutrient agar medium and following incubation and cell growth at 30°C, a visual examination did not indicate any contamination.

Master Flasks were grown as needed by inoculating 1000 ml of medium containing 1% v/v octane with one 3 ml

stock ampoule at 30°C and 290 rpm until cell growth began to enter the stationary phase. (Fig. 1). The master flask, with an optical density of 12 at 660 nm, was stored at 4°C for no longer than 10 days.

For large scale cell growth, a 14 liter New Brunswick Model 19 fermentor equipped with dissolved oxygen and pH probes and a mechanical foam breaker was utilized. 10 liters of medium containing 0.1% v/v polypropyleneglycol as an antifoaming agent and 100 ml octane, was inoculated with 300 ml from a freshly prepared master flask. During the course of the fermentation, the pH was maintained between 7.0-7.2 by 5N NaOH addition, and the temperature maintained at 30°C. Care was taken to control the air sparging and octane concentration in order to minimize toxicity to the cells and substrate evaporation. The cells were harvested during the late log phase, (Fig. 2), then centrifuged and stored frozen at 0°C. Approximately 350-400 g (wet weight) of highly active cells were obtained for every fermentation.

Epoxide Assays

Growing Cultures

To a 300 ml baffled shake flask containing 100 ml P-1 medium were added 1 ml each of octane and 1,7-octadiene and a 2 ml inoculum. The reaction mixture was incubated at 30°C. During the incubation period, cell growth occurred at the expense of octane, and 1,7-octadiene

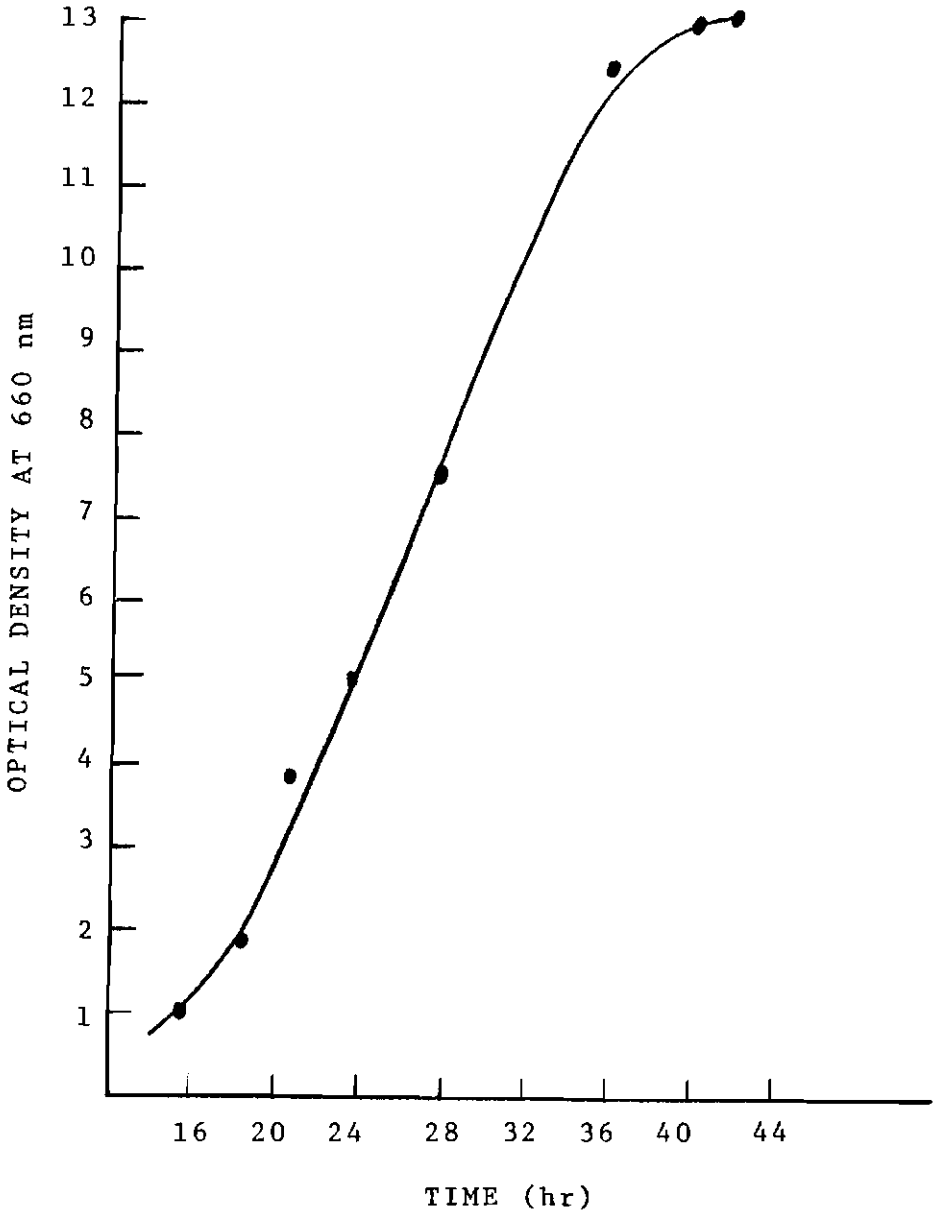


Figure 1. Growth Curve Relating Optical Density at 660 nm to the Incubation Time for a Master Flask Preparation from a Stock Ampoule

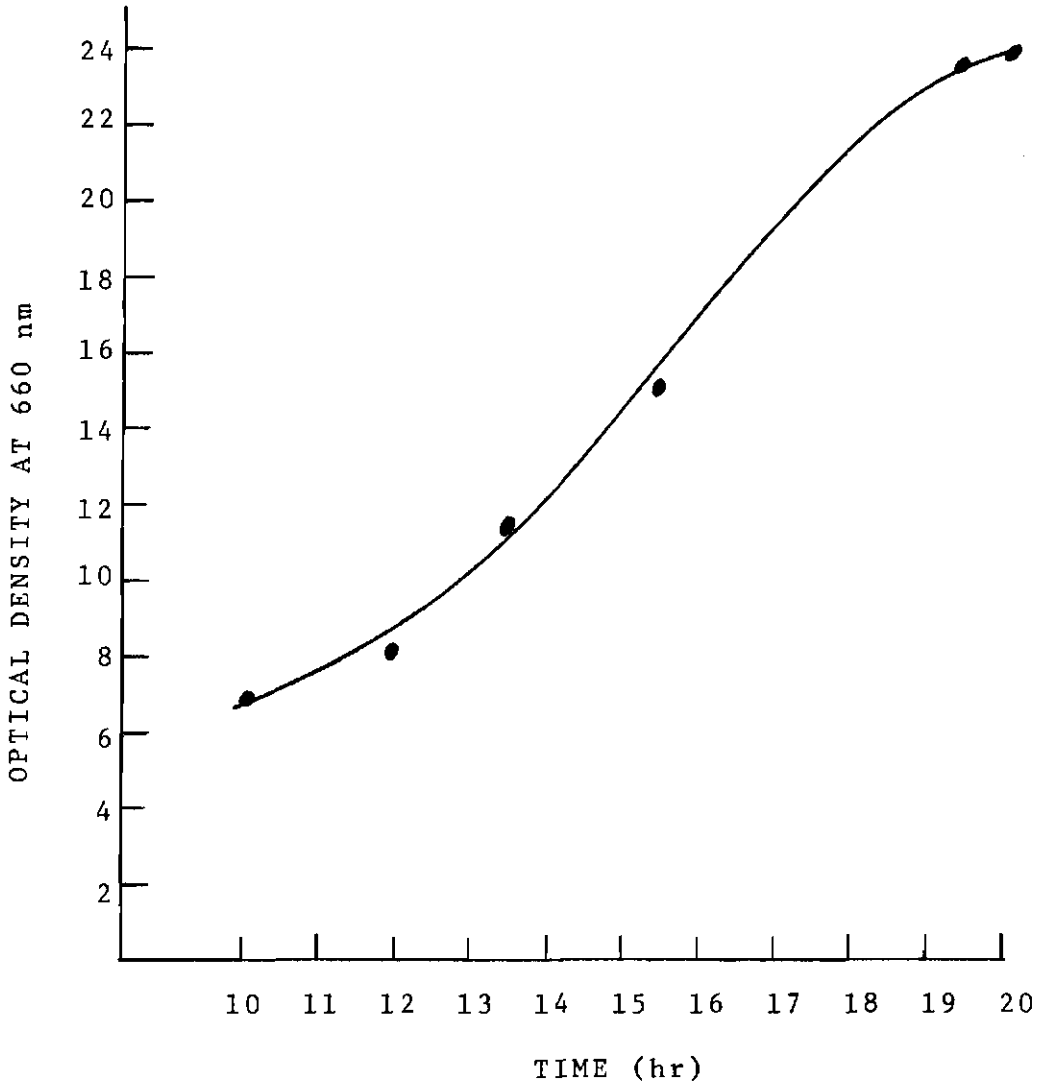


Figure 2. Growth Curve Relating Optical Density to Incubation for a 10 Liter Fermentation of P. Oleovorans

was epoxidated. At various time intervals, aliquots of the reaction mixture were taken and analyzed for both epoxidation activity and cell growth.

Resting Cell Suspensions

To a 3-liter flask containing 1000 ml P-1 medium were added 10 ml octane and a 30 ml inoculum. Incubation was carried out until the cell suspension approached an OD = 3 at 660 nm. Centrifugation at 20,000 x g and 4°C for 10 minutes gave a clear but faint yellowish supernatant, which was discarded, and reddish brown cell pellets. The cell pellets were washed twice with 0.05M phosphate buffer, pH 7.0, then resuspended in same buffer to the desired O.D. To the cell suspension were added 1% v/v 1,7-octadiene to initiate the reaction and 0.1% v/v Triton X-100 to aid in substrate solubility. The reaction mixture was stirred at room temperature and at various time intervals, aliquots were taken and assayed for epoxidation activity.

Cell-Free Preparations

Typically, to a 25% v/v suspension of whole cells in either 0.05M Tris-Cl buffer, pH 7.3 or 0.05M phosphate buffer, pH 7.0, were added 2 mg each of RNase and DNase, and 0.1% v/v mercaptoethanol. Mercaptoethanol was included to serve as a radical scavenger and therefore minimize the effects of cavitation processes during sonication. The cell suspension was sonicated at maximal output using an Artek 300 watt ultrasonic dismembrator for 10 min. but

in several bursts so as to maintain a temperature $\leq$ 15°C. (See page 43 for a discussion of the effect of sonication time). Following sonication, centrifugation at 28,000 x g and 4°C for 90 min. was required in order to effectively remove all cellular debris. After pH adjustment of the reddish brown supernatant to 7.3 with 2N NaOH, 1% v/v 1,7-octadiene and NADH (1 mg/1ml extract) were added. The reaction mixture was stirred at room temperature and periodically assayed for activity.

Product Extraction

The oxidation products of the P. oleovorans system were extracted from the aqueous solution with hexane to which 2-octanol had been added as an internal standard. The products were separated and analyzed by gas chromatography. For a quantitative estimation of the product 7,8-epoxy-1-octene, various amounts of the epoxide were added to a solution of 0.5 µl 2-octanol in 1.0 ml hexane then analyzed by gas chromatography. As illustrated in Fig. 3, a standard curve was plotted relating the concentration of 7,8-epoxy-1-oxtene to the ratio of peak areas measured for 7,8-epoxy-1-oxtene and 2-octanol. As evident by this plot, a linear relationship existed between the epoxide concentration and the ratio of peak areas. As shown in Fig. 3, a comparison of two peak area ratios from this curve to those obtained when 7,8-epoxy-1-octene was

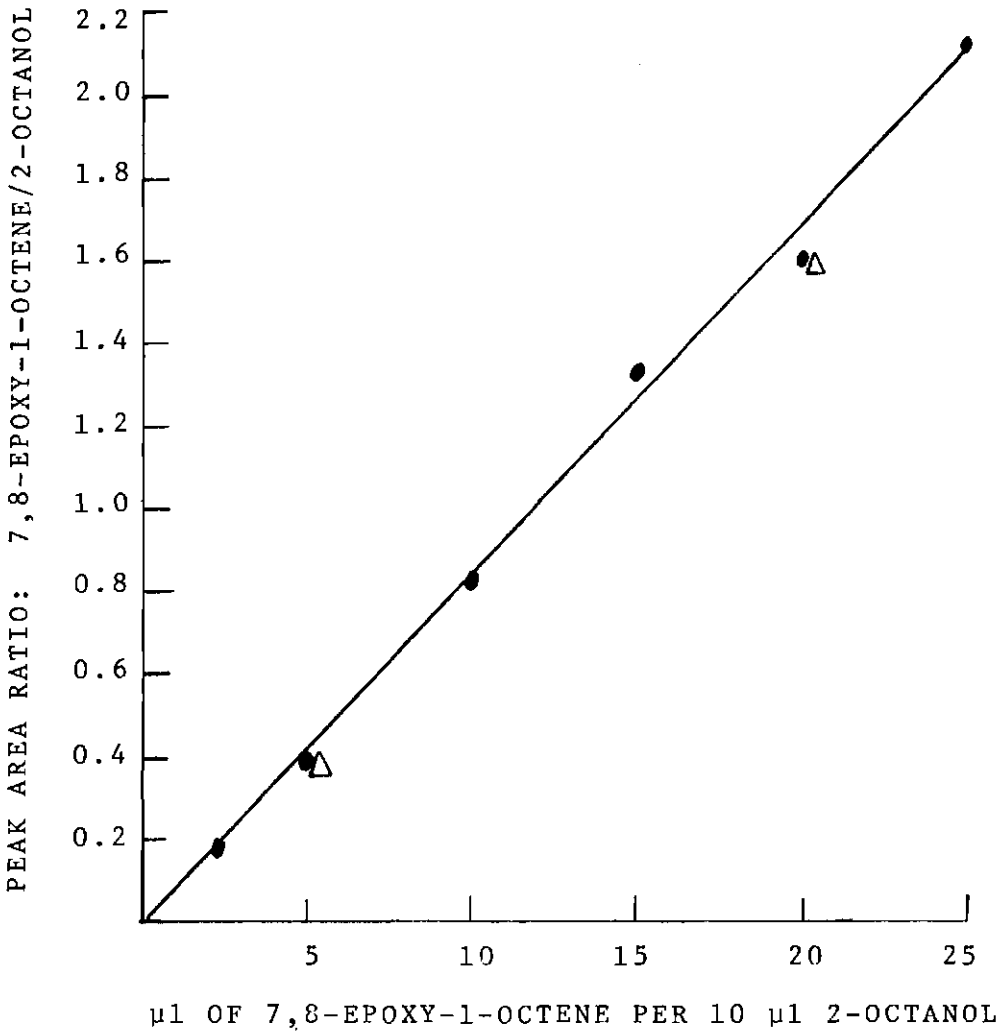


Figure 3. Standard Curve Relating the Concentration of 7,8-Epoxy-1-Octene to the Ratio of Peak Areas Measured for 7,8-Epoxy-1-Octene and 2-Octanol. 1.1 7,8-Epoxy-1-Octene Added to Hexane Solution Containing 2-Octanol. Δ: 7,8-Epoxy-1-Octene Added to Phosphate Buffer then Extracted with an Equal Volume of Hexane Containing 2-Octanol

added to phosphate buffer then extracted with 1 ml hexane containing 2-octanol indicated that essentially all of the epoxide could be recovered. For preparative work, the oxidation products were extracted from the reaction mixture using diethylether. The ether-water emulsion was broken by centrifugation and the ether layer was dried over $MgSO_4$ then concentrated. The products were separated and isolated via preparative gas chromatography.

Gas Chromatography

The reaction products were separated using a Varian Model 1700 flame ionization gas chromatograph. For analytical detection and quantitation, a stainless steel column (20 ft x 1/8 inch) packed with 10% Carbowax 20M on 80/100 Chromasorb W was used. A chromatogram which demonstrates the separation of the epoxide products and the internal standard is shown in Fig. 4. The column temperature was maintained isothermally at 175°C, the injector temperature at 220°C and the detector temperature at 250°C. The carrier gas flow rate was 30 ml of nitrogen per min., the hydrogen flow rate was 25 ml/min. and the air flow rate was 250 ml/min. For the analytical detection and quantitation of the allylic alcohols, and the saturated and α,β-unsaturated ketones, the same column and conditions were used, except that the column temperature was decreased to 155°C.

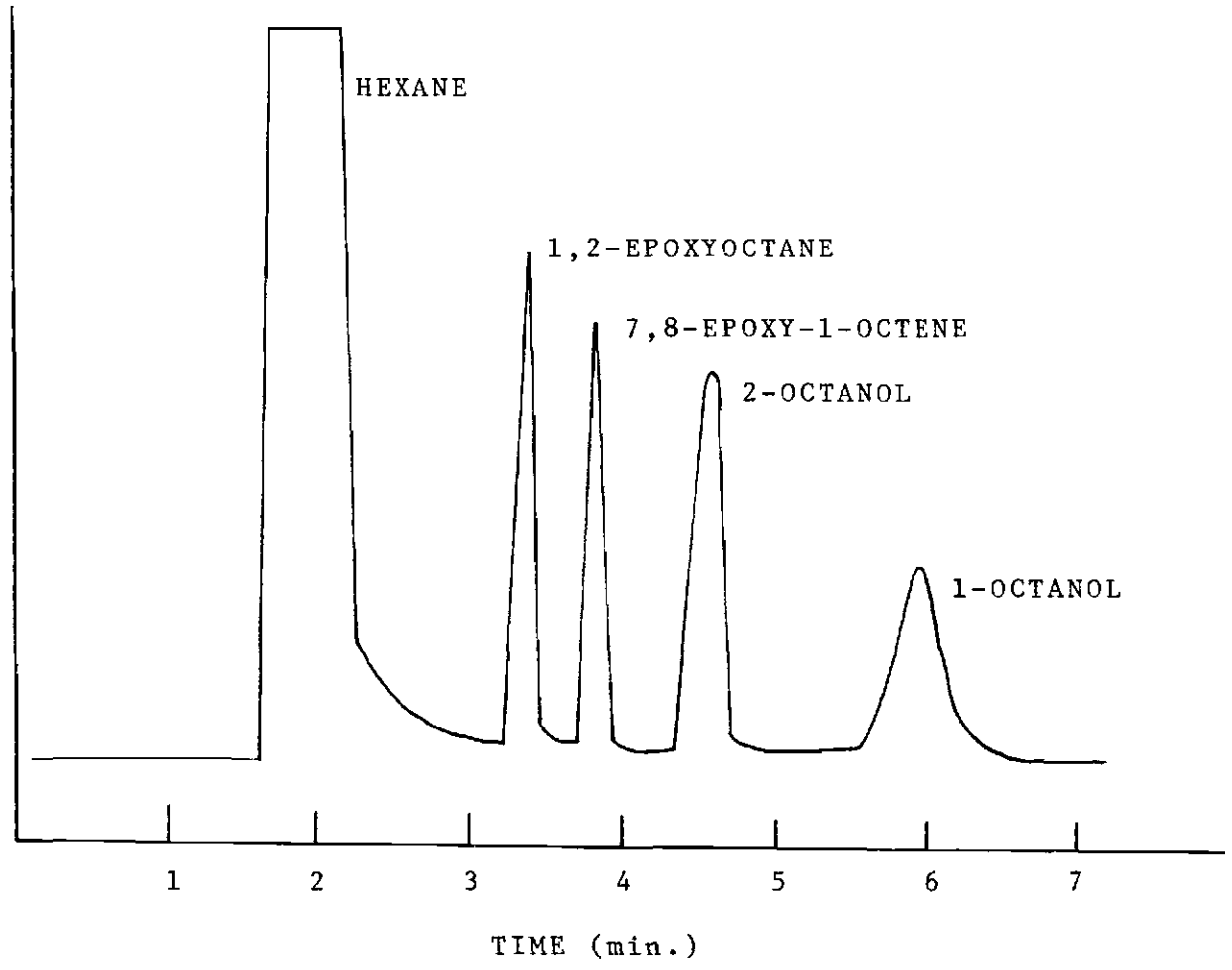


Figure 4. Chromatogram, Which Demonstrates the Separation of Epoxides from the Internal Standards, was Obtained under Conditions Described in Text

For preparative gas chromatography of all products, the same column as above but with dimensions 20 ft x 1/4 inch was used under similar conditions.

Separation and Partial Purification of the Three Protein Components

Ammonium Sulfate Fractionation

To a cell-free supernatant obtained after sonication and 90 minute centrifugation of a 25% v/v cell suspension in 0.05M Tris-Cl buffer, pH 7.3, was added solid ultrapure ammonium sulfate at room temperature to 24% saturation. During the addition of ammonium sulfate, the pH of the solution was maintained at 7.3 by 2N NaOH addition. After 10 min. centrifugation at 25,000 x g and 4°C, the precipitated fraction was discarded and solid ammonium sulfate was added to the supernatant to give the desired saturation. Each precipitated fraction was resuspended in a minimal volume of 0.02M Tris-Cl buffer, pH 7.3, and then dialyzed against the same buffer for 24 hr. with several changes. Each crude extract was concentrated on an Amicon ultrafiltration unit using a PM-10 membrane and then stored frozen with 10% v/v glycerin.

DEAE Cellulose Ion Exchange Chromatography

For the separation and partial purification of the reductase and rubredoxin components, the 24-60% ammonium sulfate fraction was resuspended and dialyzed, and then

applied to a pre-equilibrated (0.02 Tris, pH 7.4) column (5.2 x 37 cm) of Whatman DE-52 microgranular cellulose. The column was eluted successively with 0.02M Tris, pH 7.4; 0.1M Tris, pH 7.4; 0.1M KCl/0.1M Tris: and a gradient of 0.1M KCl-0.5m KCl/0.1M Tris. Partially purified reductase was eluted during the 0.1M Tris wash and partially purified rubredoxin was eluted during the salt gradient.

Biogel A 0.5M and 50M Agarose Chromatography

For the partial purification of the "epoxidase" protein, the 24-36% ammonium sulfate fraction was resuspended, dialyzed and applied to either Biogel A - 0.5M or Biogel A - 50M (Biorad). The column was eluted with 0.05M KCl/ 0.1M Tris.

The three protein components were identified on the basis of assay results and on uv-visible absorbance spectra. Assays were carried out on each of the ammonium sulfate fractions, on each as well as on combinations of the various DEAE elutions and on the various Biogel A eluted tube samples. Epoxidation reactions were with 1 ml samples to which 1% v/v 1,7-octadiene and 1 mg each of NADH and phosphatidylcholine were added. In cases of purified enzyme addition, 100 µl (0.2 mg) rubredoxin and 100 µl (0.3 mg) reductase were used.

Syntheses of Authentic Standards

Enzymatically produced compounds were identified by

retention time, proton NMR and mass spectral comparisons with authentic standards.

7,8-Epoxy-1-octene[22]

To a solution of 10 g (90.9 mmoles) of 1,7-octadiene in 150 ml diethylether was added 15.7 g (91.0 mmoles) of m-chloroperbenzoic acid, and the reaction mixture was stirred at room temperature for 17 hr. The reaction mixture was washed four times each with 20% K_2CO_3 and 10% Na_2SO_3 and twice with water then dried over $MgSO_4$. The ether extract was concentrated, then distilled to give pure 7,8-epoxy-1-octene, B.P. 59-60°C at 7.0 mm Hg. NMR (δ, CCl_4), 1.46 (m, 6), 2.05 (m, 2), 2.33 (d of d, 1), 2.56 (d of d, 1), 2.75 (m, 1), 4.85 (d of d, 1), 5.05 (d of d, 1), 5.65 (m, 1).

1,2-7,8-Diepoxyoctane[22]

The diepoxide was synthesized from 1,7-octadiene and m-chloroperbenzoic acid using the same general procedure as described for the monoepoxide except a 2:1 molar ratio of peracid to diene was used and the reaction time was increased to 40 hr. Analysis of the crude product by g.c. indicated > 95% purity. NMR (δ, CCl_4), 1.48 (m, 8), 2.33 (d of d, 2), 2.56 (d of d, 2), 2.75 (m,2).

1-Octen-3-one

1-Octen-3-one was synthesized via the Jones' oxidation[54] of 1-octen-3-ol. Jones' reagent was prepared by adding 2.67 g (0.0267 moles) CrO_3 to 2.3 ml concentrated

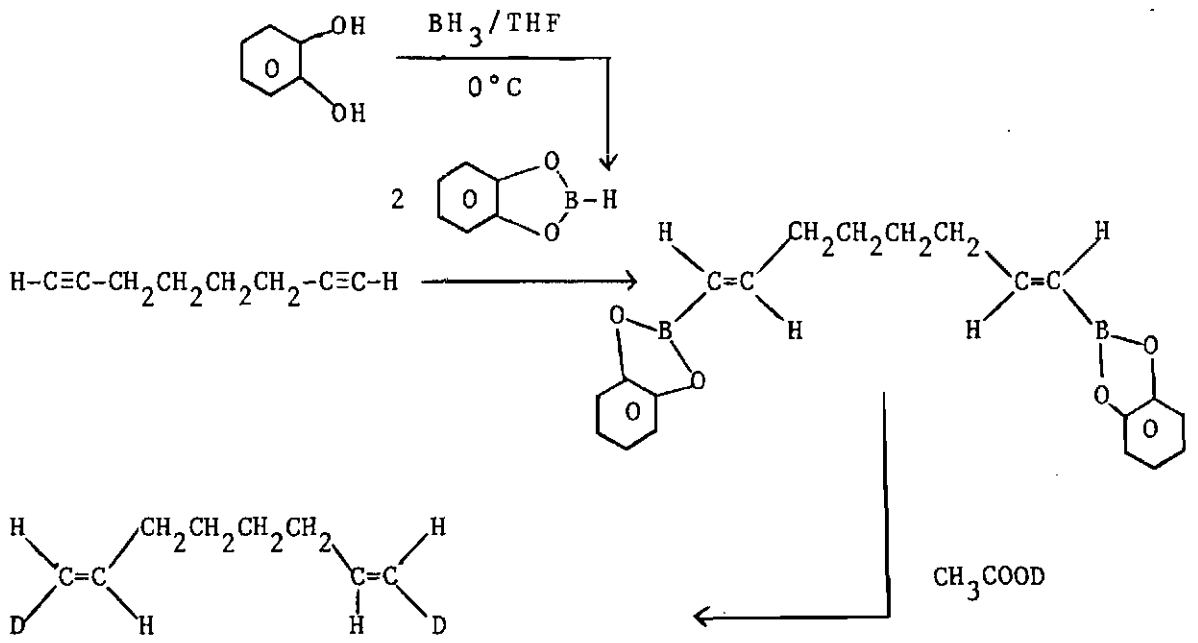
sulfuric acid and diluted slowly with water to a total volume of 10 ml. To a solution of 5 g (0.039 moles) 1-octen-3-ol and 50 ml acetone in a 250 ml round bottom flask was added dropwise at 10°C, 10 ml of Jones' reagent, (2.67 M in Cr VI). The progress of the oxidation was followed by the color change from the orange Cr VI oxidant to the green color of the hydrated chromium III product. After completion of the addition, the reaction mixture was diluted with 50 ml water then extracted with an equal volume of ether. The ether extract was washed successively with saturated $NaHCO_3$, brine, and H_2O then dried over $MgSO_4$. The ether extract was concentrated and the product isolated by preparative g.c. Yield, 4.5 g (90%), NMR (δ, CCl_4), .95 (t, 3), 1.2 (m, 6), 2.25 (m, 2), 5.2 (d of d, 1), 5.2 (m, 2). Mass spectrum: m/e = 55 (100%).

2-Methyl-1-hepten-3-one

2-Methyl-1-hepten-3-one was synthesized via the Jones' oxidation of 2-methyl-1-hepten-3-ol using the same procedure as described above. The product was isolated by preparative g.c. Yield, 4.0 g (81%), NMR (δ, CCl_4), .94 (t, 3), 1.2 (m, 4), 1.75 (d, 3), 2.62 (m, 2), 5.82 (d, 2). Mass spectrum: M^+ = 126 (4%), m/e = 69 (100%).

Syntheses of Trans,trans-1,8-dideuterio-1,7-octadiene

The deuterated diene was synthesized according to the following reaction scheme:



1,3,2-Benzodioxaborole (catecholborane)

Four hundred ml of a 1M solution of diborane in THF (Aldrich) was poured into a 3-necked round bottom flask. The solution was maintained under a nitrogen atmosphere at 0°C. A solution of 44 g (0.40 moles) of pyrocatechol (recrystallized from hot benzene, m.p. 105°C) and 50 ml of THF (distilled over $NaAlH_4$) was added dropwise over a 30 min. period with efficient stirring. The solution

was stirred for an additional 30 min. at 0°C and then for one hr. at room temperature. THF was removed under vacuum (50 mm Hg, 25°C) and the crude product was distilled. Yield, 29.3 g catecholborane (51%); b.p., 88°C at 156 mm; 66°C at 80 mm [Lit: 88° at 156 mm].[56]

Bis-(1,7-Octadienyl-)-1,3,2-benzodioxaborole

12.45 g (0.117 moles) of freshly distilled 1,7-octadiene and 28.5 g (0.237 moles) of catecholborane were injected via syringe into a 100 ml 3-necked flask to which a reflux condenser had been attached. The apparatus had been flame dried and cooled under N_2 before use. After several minutes of stirring, the temperature of the reaction mixture rose to 110°C. Stirring was continued for 1 hr. after which a white solid was formed. The crude product was recrystallized from ethyl acetate and dried under vacuum. Yield, 22.3 g (55%); m.p., 96-98°, NMR (δ, CD_3COCD_3), 1.0 (m, 4), 1.5 (m, 4), 5.2 (d, 2), 5.9 (m, 2), 6.4 (d of d, 8). Exact mass determination: 345.14532. The NMR splitting pattern indicated only trans olefinic protons (J = 18 Hz) corresponding to cis addition of the catecholborane.

trans,trans-1,8-Dideuterio-1,7-octadiene

25 g (0.409 moles) CH_3COOD (Aldrich) which consisted of $\approx$ 3-5% CH_3CO_2H, was injected via syringe into a 3-necked flask containing 15 g (0.043 moles) of the octadienylborole. The flask, fitted with a reflux condenser, had been flame

dried and cooled under N_2 before use. During the addition and throughout the course of the reaction, a nitrogen atmosphere was maintained. After three hours of reaction, the mixture was poured over 50 ml of ice water, extracted with 200 ml of pentane, washed successively with ice cold 1N NaOH and brine, and then dried over $MgSO_4$. The crude product was distilled to give 5.5 ml of the deuterated product (90% yield), b.p. 62° at 140 mm.

The final product was characterized as follows: Gas chromatographic analysis gave a single peak with retention time identical to that obtained with an authentic sample of 1,7-octadiene. Mass spectral analysis gave a molecular ion peak at m/e = 112. A large peak at m/e = 96 was also observed corresponding to allylic rearrangement and cleavage of a terminal CH_2D fragment (m/e = 16). Correspondingly, the mass spectrum of nondeuterated 1,7-octadiene showed a large peak at m/e = 95, corresponding to the removal of a terminal CH_3 fragment. The NMR was fully consistent with the structure and the relaxation spectrum analysis showed that no cis-deuterated olefin was present (see Results section).

Synthesis of cis and trans-3,4-epoxyhexane

Starting from pure samples of each of the isomeric internal olefins cis and trans-3-hexene (Chemical Samples), the configurations of which were confirmed by NMR analysis,

the compounds cis-3,4-epoxyhexane and trans-3,4-epoxyhexane were synthesized by m-chloroperbenzoic acid epoxidation. To two solutions, each containing 1 g (0.012 moles) of an isomeric olefin in 15 ml diethylether, were added 2.07 g (0.012 moles) of m-chloroperbenzoic acid. Each reaction mixture was stirred at 15°C for 20 hr., then washed successively with 20% K_2CO_3, 10% Na_2SO_3, and water. The ether layers were dried over $MgSO_4$ then concentrated. The products were isolated by preparative g.c. yielding approximately 200 mg of each epoxide. Mass spectral analysis gave a molecular ion peak of 100 for both products. cis-3,4-epoxyhexane: NMR (δ, CCl_4), .98 (t, 6), 1.40 (m, 4), 2.65 (m, 2). trans-3,4-epoxyhexane: NMR (δ, CCl_4), .92 (t, 6), 1.40 (m, 4), 2.45 (m, 2).

Epoxidation of trans,trans-1,8-dideuterio-1,7-octadiene

Two 2-l shake flasks, each containing 1000 ml of P-1 medium, 10 ml octane, and a 20 ml inoculum from a freshly prepared master flask were incubated at 30° for 17 hr. The two flasks had an O.D. at 660 nm of 6.4 and 6.2 respectively. The solutions were centrifuged then resuspended in 0.1M phosphate buffer, pH 7.0, to a total of 300 ml. One drop of Triton X-100 and 3 ml of the deuterated substrate were added and the mixture was incubated with shaking at 30°. Production of epoxide was continuously monitored by g.c. and when epoxide production was maximal,

the product was extracted with 300 ml hexane, concentrated to 4 ml, and purified by preparative g.c.

Peracid epoxidation was effected by mixing 1 g (57 mmoles) of m-chloroperbenzoic acid and 0.583 g (52 mmoles) of the deuterated diene in 30 ml ether for 48 hr. at room temperature. The epoxide product (approx. 200 mg) was collected by preparative g.c.

Epoxide Incubation with P. oleovorans: Isomerization Controls

To 150 ml of a resting cell suspension in a 300 ml shake flask were added 1.5 ml trans-3,4-epoxyhexane and one drop of Triton X-100. The solution was incubated with shaking at 30° for 7 hr. After which the unreacted epoxide was extracted with ether, concentrated to 3 ml and purified by g.c. NMR analysis indicated that the recovered monoepoxide (95% recovery) consisted of only the trans isomer.

To 50 ml of a resting cell suspension were added 400 mg of the peracid epoxidation product from trans, trans-1,8-dideuterio-1,7-octadiene and one drop of Triton X-100. The reaction mixture was incubated with shaking for 3 hr. then extracted and the unreacted epoxide purified by g.c. A NMR spectra of the reisolated compound after incubation was identical to that of the starting material.

Enzymatic Epoxidation of Racemic 7,8-epoxy-1-octene[29]

One hundred 300 ml shake flasks, each containing 100 ml P-1 medium, 1 ml octane, 1 ml (87 mg) racemic 7,8-epoxy-1-octene (from the peracid epoxidation of 1,7-octadiene), and a 10 ml inoculum were incubated for 20 hr. at 30°C. The broths were then extracted with toluene and concentrated, and both the diepoxide product and unreacted 7,8-epoxy-1-octene isolated by vacuum distillation Typical recoveries (before distillation) from a single shake flask were 30-40 mg of unreacted monoepoxide and 15-20 mg of diepoxide. The purity of each product was determined by NMR and gas chromatographic analysis. The monoepoxide was essentially 100% pure and the diepoxide was greater than 95% pure. The impurity being the unreacted monoepoxide.

Incubation of 1-octen-3-ol, 1-octen-3-one, 3-octanol 2-methyl-1-hepten-3-ol and 2-methyl-1-hepten-3-one with P. oleovorans

The enzymatic incubation of the various substrates were carried out separately using both cell-free preparations and partially purified enzymes.

For cell-free incubations, 600 µl of substrate and 6 mg NADH were added to 60 ml of cell free extract. Incubations were carried out at room temperature with shaking, and product formation was monitored by periodic extraction and g.c. analysis. Once product formation was

maximal, each reaction mixture was extracted with ether and centrifuged to break the ether-water emulsion. The ether layers were concentrated and the products isolated by preparative g.c.

For reactions involving purified enzymes, 20 µl of substrate was added to a solution of 2 ml 0.05M Tris, pH 7.3, containing 2 mg each of NADH and phosphatidylcholine, 200 µl (0.4 mg) reductase, 100 µl (0.2 mg) rubredoxin and 200 µl (0.6 mg) "epoxidase".

The products isolated from the enzymatic reactions of 1-octen-3-ol and 3-octanol were both identified to be 3-octanone on the basis of g.c. retention times, and comparison of NMR and mass spectra to those of authentic samples. 100 mg and 150 mg of 3-octanone were isolated from the cell free incubation of 1-octen-3-ol and 3-octanol respectively. Similarly, the product from the incubation of 2-methyl-1-hepten-3-ol was identified to be 2-methyl-3-heptanone with 30 mg of product isolated.

On the other hand, yields from the reactions of 1-octen-3-one and 2-methyl-1-hepten-3-one were low and the products were not isolated. On the basis of retention time comparisons only, the products formed from 1-octen-3-one and 2-methyl-1-hepten-3-one were apparently the corresponding saturated ketones.

3-octanone from 1-octen-3-ol; NMR (δ, $CDCl_3$), 1.0 (m, 6), 1.2 (m, 6), 2.2 (m, 4). Mass spectrum: $M^+ = 128$

(30%), m/e = 43 (100%).

3-octanone from 3-octanol; NMR (δ, $CDCl_3$), 1.0 (m, 6), 1.2 (m, 6), 2.2 (m, 4). Mass spectrum: M^+ = 128 (15%), m/e = 43 (100%).

2-methyl-3-heptanone from 2-methyl-1-hepten-3-ol; NMR (δ, CCl_4), .64 (d, 6), .98 (t, 3), 1.40 (m, 4), 2.25 (m, 3). Mass spectrum: M^+ = 128 (15%), m/e = 57 (100%).

Partially Relaxed NMR Procedures

Sample Preparations

100 mg of the enzymatic epoxidation product of trans, trans-1,8-dideuterio-1,7-octadiene was dissolved in 0.25 ml CCl_4 which contained 10% v/v C_6D_6 for the deuterium lock signal. The solution was made up in a standard 5 mm o.d. NMR sample tube which was fitted with a ground glass joint for attachment to a high vacuum manifold. The oxygen was removed by repeated freeze-pump-thaw cycles using a dry ice-acetone bath as the coolant.

Instrumentation

FT NMR proton spectra were obtained at 99.5 MHz using a JEOL PFT-100 FTNMR spectrometer equipped with a PG-100 computer system. The observed frequency was derived from a general radio 1164-A frequency synthesizer which was phase locked to the 15.4 MHz D lock frequency.

Partially Relaxed Spectra

The partially relaxed spectra were obtained using a 180°-τ-90° pulse sequence.[57] The width of the 90° pulse

was 55 μ sec., corresponding to an rf field of amplitude 4.7 KHz. An 8K transform was used with a window of 1 KHz. Each spectrum was the result of 16 accumulations with a repetition time between pulse sequences of 100 sec. This repetition is 3.4 times the longest relaxation time of the sample. As a check, spectra were also obtained with a repetition time of 200 sec. No systematic difference in spectral intensities were observed between these spectra and those with a repetition time of 100 sec.

A sequence of partially relaxed spectra were obtained in this manner with the interval time stepped from 1 sec. to 25 sec. in 1 sec. steps. This enabled the null time in most cases to be determined to within $\pm$ 1 sec. When justified, smaller increments of τ were used to determine the null time more accurately. After the null times were determined, three spectra were obtained at each null time. In addition, three spectra were obtained with τ = 0.2 sec. and τ = 100 sec. in order to determine the fractional inversion produced by the 180° pulse. The spectra were obtained over a period of 6 days. During this time, the sample remained continuously in the NMR probe. The only spectrometer adjustments during the period were to the curvature and Y gradient of the field homogeneity unit.

The spectra were recorded on chart paper, and the intensities of the relevant spectral features were measured with a Gelman Model 39231 compensating polar planimeter.

In general, the intensities of corresponding spectral features were reproducible to better than 10% and in most cases to better than 5% (See Table 3, Results section).

Optically Active NMR Shift Studies of Enzymatically Produced Epoxides

The optical purities of the enzymatically produced 7,8-epoxy-1-octene and 1,2-7,8-diepoxyoctane were determined by NMR using a chiral shift reagent and compared to those determined for the chemically produced epoxides. The chemically produced 7,8-epoxy-1-octene and 1,2-7,8-diepoxyoctane were prepared by the peracid epoxidation of 1,7-octadiene and the enzymatically produced 7,8-epoxy-1-octene was prepared from the incubation of 1,7-octadiene with a resting cell suspension of P. oleovorans as previously described. The enzymatically generated 1,2-7,8-diepoxyoctane was obtained from Dr. R. D. Schwartz, Corporate Research Laboratories, Exxon Research and Engineering, Linden, N.J.

To a solution of 32.6 mg (0.258 mmoles) of chemically synthesized 7,8-epoxy-1-octene in 0.5 ml CCl_4 were added successively 0.01, 0.01, 0.004, and 0.004 mmoles of tris-[3-(trifluoromethylhydroxymethylene)-d-camphorato]europium III. After each addition of the europium shift reagent, a 60 MHz NMR spectra was recorded. The clear separation of two multiplets corresponding to the R and S isomer was achieved with a total addition of 0.024 mmoles shift

reagent corresponding to 0.09 moles reagent per mole of epoxide. Lesser amounts of the shift reagent could not clearly separate the two multiplets whereas greater amounts resulted in overlapping of the multiplets with the olefinic signals. To minimize the noise to response ratio, a 100 MHz NMR spectra was recorded for the sample containing 0.024 mmoles shift reagent.

To a solution of 32.6 mg (0.258 mmoles) of the enzymatically produced monoepoxide in 0.5 ml CCl_4 was added 0.024 mmoles of the europium shift reagent and a 100 MHz NMR spectra recorded.

To both the chemically and enzymatically synthesized diepoxides, 0.1188 mmoles of the europium shift reagent was added to a solution of 56 mg (0.395 mmole) diepoxide in 0.5 ml CCl_4 (0.30 moles of reagent per mole diepoxide). Because of the absence of any olefinic protons in the diepoxides, greater amounts of the reagent could be added without the overlapping of any signals. A 60 MHz NMR spectrum was recorded for each sample. For a quantitative determination of the enantiomeric multiplets, spectra were recorded with a sweep width of 100 Hz and the relative areas determined from the average of 10 determinations using a planimeter. Average deviations never exceeded about 2%.

Optical Rotatory Dispersion Studies

ORD curves were obtained of neat samples of the

enzymatically produced 7,8-epoxy-1-octene and 1,2-7,8-diepoxyoctane, and both the enzymatically produced diepoxide from racemic 7,8-epoxy-1-octene and the unreacted mono-epoxide. The baseline for all measurements were set with neat samples of the chemically synthesized epoxides and a neat sample of acetone. All spectra were obtained at 25°C using a 0.1 mm pathlength cell and a JASCO ORD/UV-5 spectrophotometer.

Oxygen Requirement Studies

The rate of oxygen consumption during enzymatically catalyzed reactions was determined using a YSI 5331 oxygen probe at 30°C. The instrument was calibrated before use by measuring the decrease in oxygen saturation which accompanied the complete oxidation of a known concentration of protocatechuic acid by protocatechuate-3,4-dioxygenase.

The reactions were carried out as follows. To 3 ml of 0.05M Tris buffer, pH 7.3, were added 100 µl (0.2 mg) rubredoxin, 10 µl (0.03 mg) reductase, 100 µl (0.3 mg) "epoxidase", 100 µl NADH (14.2 mg/ml buffer), 0.1 mg d,l-α-phosphatidylcholine, and either 1 mg 1,7-octadiene or 1 mg 1-octen-3-ol.

Spectrophotometric Studies Under Anaerobic Conditions

The rate of either NADH oxidation or NADH formation was determined spectrophotometrically under anaerobic conditions for several reductase catalyzed ketonization reactions.

In order to determine whether purging of an aqueous solution using a Thunberg cell with nitrogen gas was sufficient to develop anaerobic conditions, the following test was carried out. 40 µl of a 12.2 mM solution of protocatechuic acid was added to 2.3 ml of 0.05M Tris buffer, pH 8.3, and this solution was then dispensed into a cuvette of a Thunberg cell. The cuvette was fitted with a hollow stopper which contained 1 µl of a stock solution (1.5 mg/ml) of protocatechuate-3,4-dioxygenase. Prepurified dry nitrogen was slowly bubbled through the solution in the cuvette for 30 min. at ice bath temperature and then for an additional 30 min. at room temperature. The gas was allowed to escape through a valve on the hollow stopper. The Thunberg cell was then sealed and the reaction was initiated by thoroughly shaking the cell. Oxidation of protocatechuic acid was not observed spectrophotometrically under these conditions. When air was introduced to the solution with stirring, a sharp decrease in absorbance at 290 nm with respect to time occurred, corresponding to the oxidation of protocatechuic acid. This result indicated that anaerobic conditions were obtained using this procedure.

For the enzymatically catalyzed ketonization reactions, the rate of either NADH oxidation or NADH formation was determined by measuring the change in absorbance at 340 nm. To 2.3 ml 0.05M Tris, pH 7.3 in a Thunberg cuvette were added 15 µl of a stock solution

(14.2 mg/ml) of either NADH or NAD^+, and 5 µl (0.01 mg) reductase. To the hollow stopper was added 3 µl of substrate (1-octen-3-ol, 1-octen-3-one or 3-octanol). The Thunberg cell was purged with N_2 and the reaction initiated as described above.

CHAPTER III

RESULTS

Epoxidation of 1,7-octadiene by Cells Growing on Octane

In a system involving an aqueous minimal salts medium inoculated with P. oleovorans and containing both octane (1% v/v) and 1,7-octadiene (1% v/v), cell growth occurs at the expense of octane, and 1,7-octadiene is epoxidated. Octane is required in this system since 1,7-octadiene, which lacks a terminal methyl group, cannot support cell growth.[58,59] 7,8-Epoxy-1-octene is the major product detected by gas chromatography with conversions of octadiene to the monoepoxide approaching 5% or a product yield of 0.5 mg/ml, for a reaction time of about 14 hr. (Figure 5). Other oxidation products detected but in negligible amounts are 1-octanol and 1,2-7,8-diepoxyoctane. In general, a maximal concentration of 7,8-epoxy-1-octene is obtained several hours after cell growth enters a stationary phase. As shown in Figure 5, an examination of the time course of epoxide formation revealed that the epoxide concentration decreases during prolonged incubation. A decrease by as much as 50% is frequently observed within several hours after conversions reach a maximum. Although

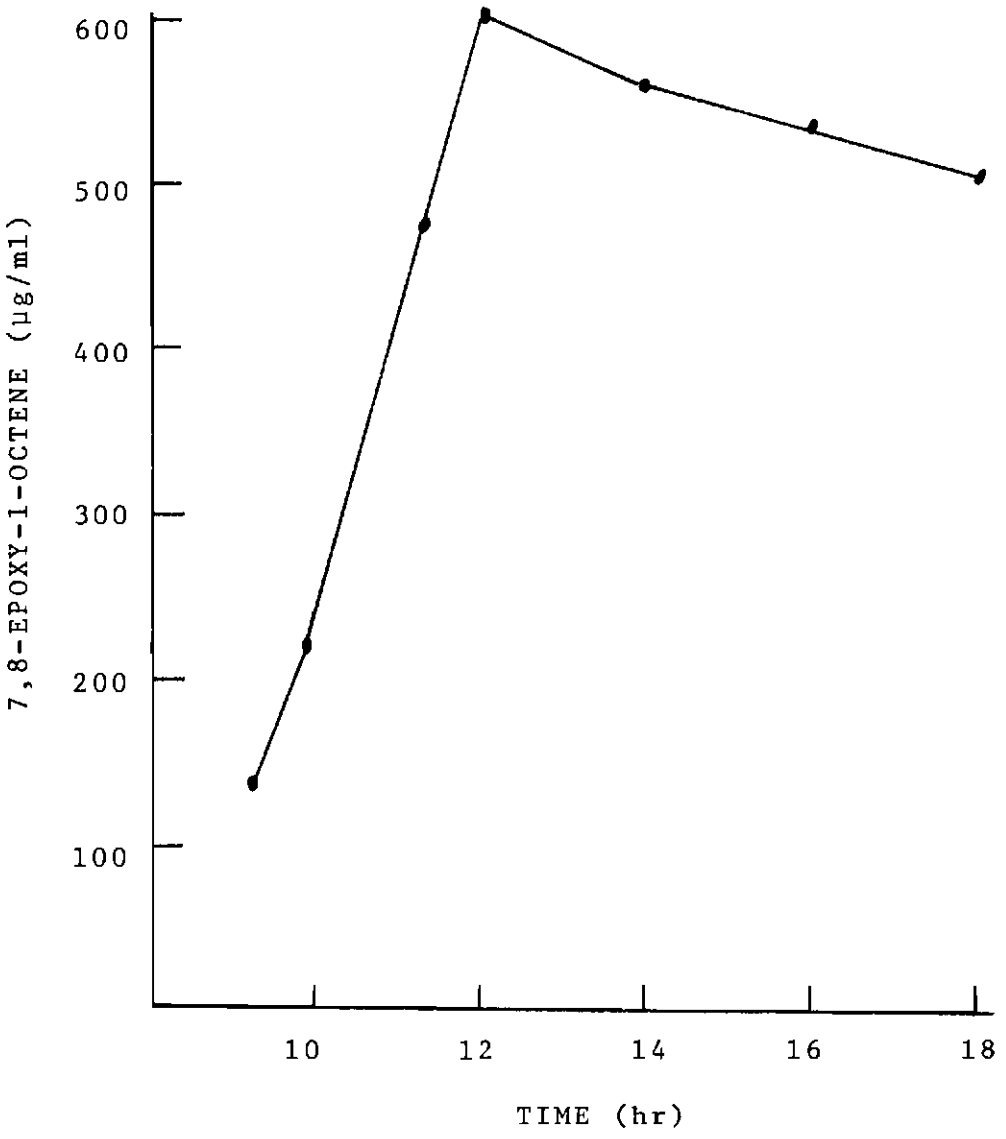


Figure 5. Epoxidation of 1,7-octadiene by Cells Growing on Octane. To 100 ml of P-1 medium was added 1% v/v 1,7-octadiene and a 2 ml inoculum. Incubation was at 30° and 290 rpm.

the synthesis of 1,2-7,8-diepoxyoctane occurs, diepoxide formation alone cannot account for the epoxide loss.

Substrate concentrations within 0.5-1% v/v are optimal for maximal product yields, whereas concentrations of 5% or greater results in limited cell growth and significantly lower yields. A similar effect of substrate concentration of product yields is observed in the epoxidation of 1-octene by growing cultures of P. oleovorans.[58] Larger volumes of inoculum, however, results in only shorter incubation times for both maximal cell growth and maximal conversions.

Epoxidation with Resting Cell Suspensions

Figure 6, illustrates the results obtained for the conversion of 1,7-octadiene to 7,8-epoxy-1-octene with resting cell suspensions of P. oleovorans and establishes the feasibility of producing epoxides using whole cells. Inspection of the figure reveals that our best yields approach 2 mg of epoxide per ml of reaction mixture, or a 20% conversion, which is several fold higher than reported previously.[23,59] These data underscore one of the most significant advantages of the enzymatic epoxidation for mechanistic and stereochemical studies, since relatively large amounts of products can be readily generated. As evident in Figure 6, a considerable decrease in epoxide concentration occurs once synthesis of 7,8-epoxy-1-octene

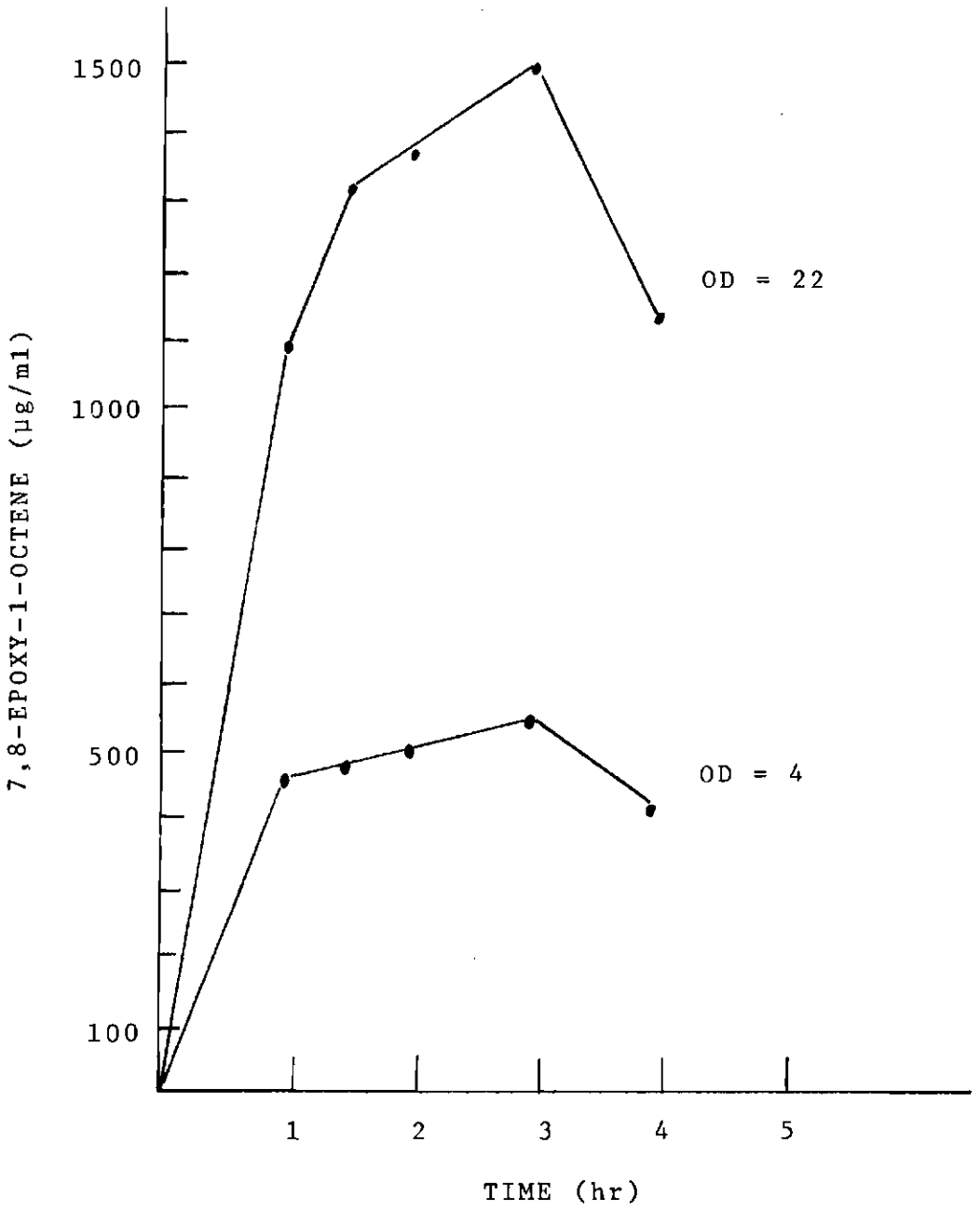


Figure 6. Production of 7,8-epoxy-1-octene from 1,7-octadiene by a Resting Cell Suspension. To each suspension was added 1% v/v substrate and 0.1% v/v of Triton X-100.

reaches a maximum. 1,2-7,8-diepoxyoctane is the only other product detected by gas chromatography, but diepoxide formation is negligible and clearly cannot account for the significant loss of the monoepoxide. One of the limiting factors which prevents obtaining even higher product yields with whole cells is the toxic effect of 7,8-epoxy-1-octene on whole cells of P. oleovorans. Schwartz and McCoy[60] have reported that in aqueous medium, 7,8-epoxy-1-octene is toxic to the cells at a concentration of about 0.8 g/1. They report that a higher efficiency in the conversion of 1,7-octadiene to 7,8-epoxy-1-octene using growing cultures can be achieved by the incorporation of a high concentration of an organic solvent to the reaction medium.[61] Our preliminary investigations with the incorporation of a 30% v/v cyclohexane into a resting cell suspension, indicate an increase in conversions of about 20%; however, incubation times of > 50 hr. are required.

Epoxidation with Cell-Free Extracts

As described in the experimental section, cell-free preparations of P. oleovorans are prepared by subjecting a resting cell suspension to sonication. The effect of the sonication treatment was studied so as to maximize the release of enzymes into solution as well as to maintain the stability of the three component enzyme system. As shown in Figure 7, a sonication time of 10 minutes for a 25%

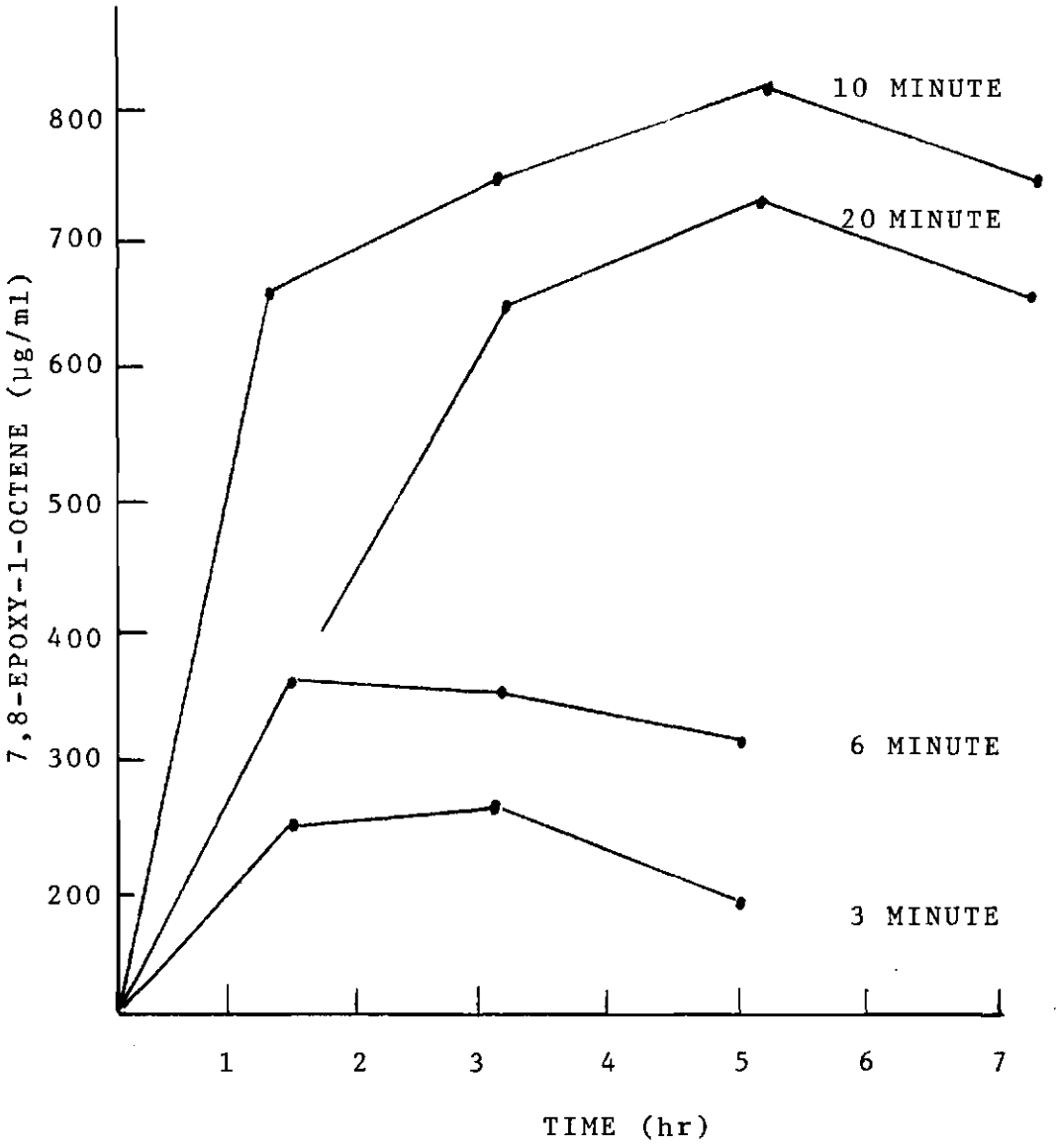


Figure 7. Epoxidation Assays of Supernatants Obtained After Sonication of a 25% v/v Resting Cell Suspension

v/v of a resting cell suspension is optimal in providing a maximal product yield of 1 mg/ml for the supernatant. Longer sonications result in lower yields, possibly reflecting the denaturing effect of a too severe treatment. The lower yields with shorter sonications indicate the ineffectiveness of cell breaking and the subsequent release of enzymes into solution. This is further indicated by the data in Figure 8, which demonstrates that higher activity is associated with the residual matter of shorter sonications.

The sonication treatment not only provides for the release of all three protein components into solution, but also the release of NADH and phospholipids. The addition of NADH to the sonicate is not required for epoxidation activity, nor does it provide any significant stimulatory effect; however, the addition of NADH does minimize the decrease in epoxide concentration which occurs with prolonged incubation. The release of lipids is found to be dependent upon the severity of the sonication treatment. Lipid addition provides a significant stimulatory effect to those solutions of short sonications, but has no effect upon solutions of 10 min. or greater sonications. Although a stimulatory effect is observed, the presence of lipids is not required for epoxidation activity in cell free extracts. A stimulatory effect of lipid addition has been

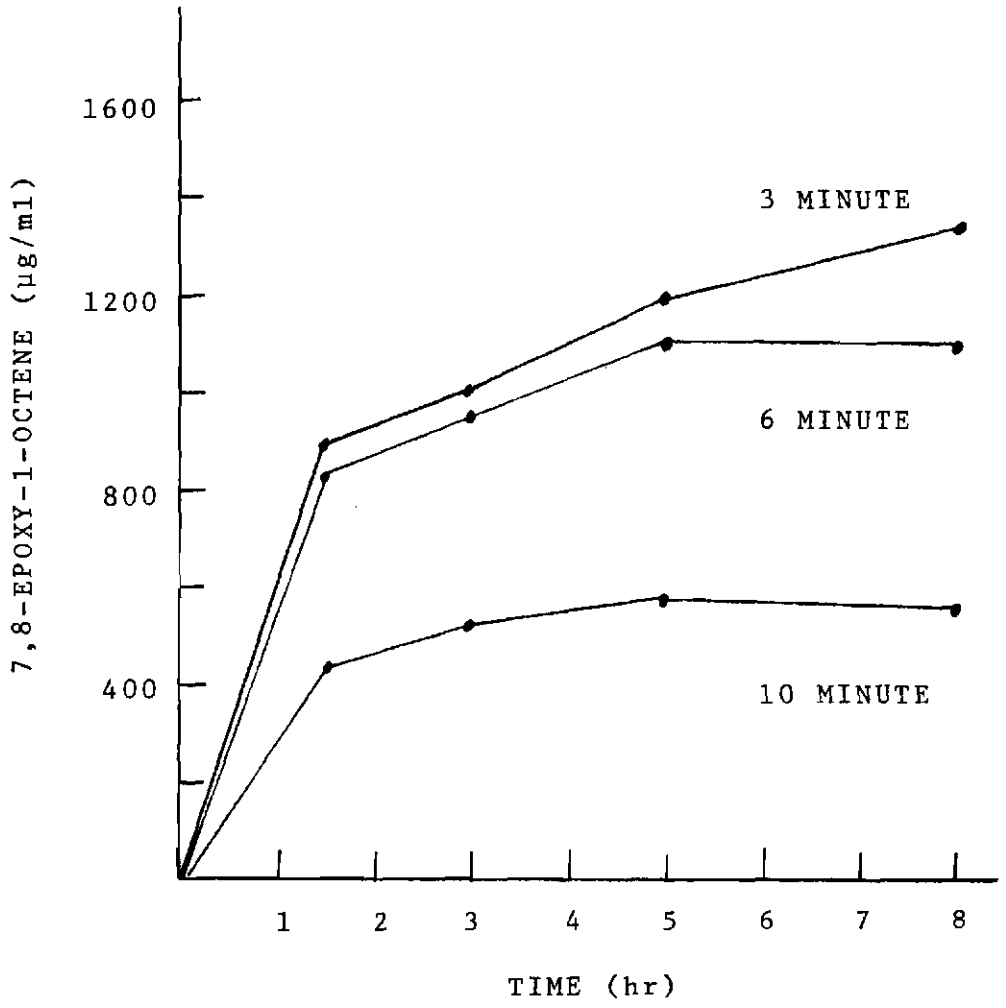


Figure 8. Epoxidation Assays of Residual Matter Obtained after Sonication of a 25% v/v Resting Cell Suspension and Centrifugation. The residual matter was resuspended in buffer containing several drops (.1% v/v) of Triton X-100.

shown for the hydroxylation reaction catalyzed by the *P. oleovorans* system.[12]

Ammonium Sulfate Fractionation

As previously described, reactions involving whole cells or crude cell-free sonicates provide for the syntheses of epoxides from simple olefins on a preparative scale. To further explore the usefulness and versatility of this system in organic chemistry, it was desirable to develop simple procedures for obtaining partially purified enzymes with high epoxidation activity. Ammonium sulfate fractionation of a crude cell-free sonicate was an initial approach to develop a single, facile procedure for separating all three protein components.

Various ammonium sulfate fractions, after resuspension, dialysis, and concentration, were assayed for epoxidation activity. The results of these assays are presented in Table 1. Since epoxidation activity requires the presence of all three protein components, the results indicate that rubredoxin, reductase and "epoxidase", all precipitate within an ammonium sulfate cut of 25-55%. In the presence of NADH and d,l-α-phosphatidylcholine, this fraction catalyzes the synthesis of 7,8-epoxy-1-octene from octadiene with product yields approaching 0.5 mg/ml. Unlike the epoxidation reactions using whole cells or cell sonicates, once epoxide concentration reaches a maximum

Table 1. Epoxidation Assays of Ammonium Sulfate Fractions

Saturation	7,8-epoxy-1-octene Formation
0-65%	++
0-60%	++
0-55%	++
0-50%	+
37-55%	-
25-55%	++
0-25%	-
0-30%	-
25-37%	-
25-37% + 37-55%	++
25-37% + rubredoxin	+
25-37% + reductase + rubredoxin	++
37-55% + rubredoxin	-
0-25% + rubredoxin + reductase	-

Assay results of ammonium sulfate fractions. Each fraction was suspended in buffer, dialyzed and concentrated before reaction. Product 7,8-epoxy-1-octene was detected and quantitated by gas chromatography.

++: % conversions within 5-7%

+ : % conversions within 2-4%

- : no product detected

with a 25-55% ammonium sulfate fraction, prolonged reaction times result in only a negligible decrease in the mono-epoxide concentration. This is not the case, however, with fractions of saturations greater than 55%. Prolonged reaction time with a 25-60% fraction does result in a loss of monoepoxide, and the rate and extent of the loss increases as the saturation is increased to 65%. This finding suggests that the epoxide loss is an enzymatic process catalyzed by enzymes precipitating at saturations greater than 55%. An ammonium sulfate fraction of 25-37% saturation in the presence of NADH and lipid does not catalyze epoxide formation, but upon addition of purified rubredoxin, epoxide formation results with a product yield of about 0.2 mg/ml. With the addition of both purified reductase and rubredoxin to a 25-37% fraction, the product yield increases to 0.5 mg/ml. These results indicate that the 25-37% fraction is rich in "epoxidase" and contains some reductase but does not contain rubredoxin.

A 37-55% fraction does not show epoxidation activity by itself or in the presence of rubredoxin and reductase, but when combined with a 25-37% fraction, activity is observed. This fraction, therefore, is apparently rich in rubredoxin and possibly reductase but does not contain "epoxidase".

A fraction of 25% saturation does not contain any of the three protein components. This fraction shows no

activity when combined with either a 25-37% or a 37-55% fraction, or in the presence of rubredoxin and reductase. In addition, this fraction does not provide any stimulatory effect when combined with a 25-55% fraction.

Further refinements in the ammonium sulfate fractionation procedure did not improve on the separation of the three protein components. Ammonium sulfate fractionation, at best, provides for the separation of "epoxidase" from rubredoxin but is not effective in separating reductase from "epoxidase" or possibly from rubredoxin.

Ion Exchange Chromatography on DEAE Cellulose

An elution profile associated with DEAE chromatography of the 24-60% ammonium sulfate fraction is shown in Figure 9. The 0.02M Tris buffer wash involves the elution of non absorbing proteins. A high concentration of proteins are eluted as evidenced by the high absorbance at 280 nm. In addition to the typical protein absorbance at 280 nm, spectral properties of this effluent include a sharp absorbance at 410 nm. From the 0.1M Tris wash, two peaks were detected. Peak B was dark yellow and exhibited a sharp absorbance at 410 nm whereas Peak C was light yellow and exhibited significant broad absorbances at 370 nm and 450 nm. These absorbances at 370 nm and 450 nm are characteristic features of flavoproteins, suggesting that

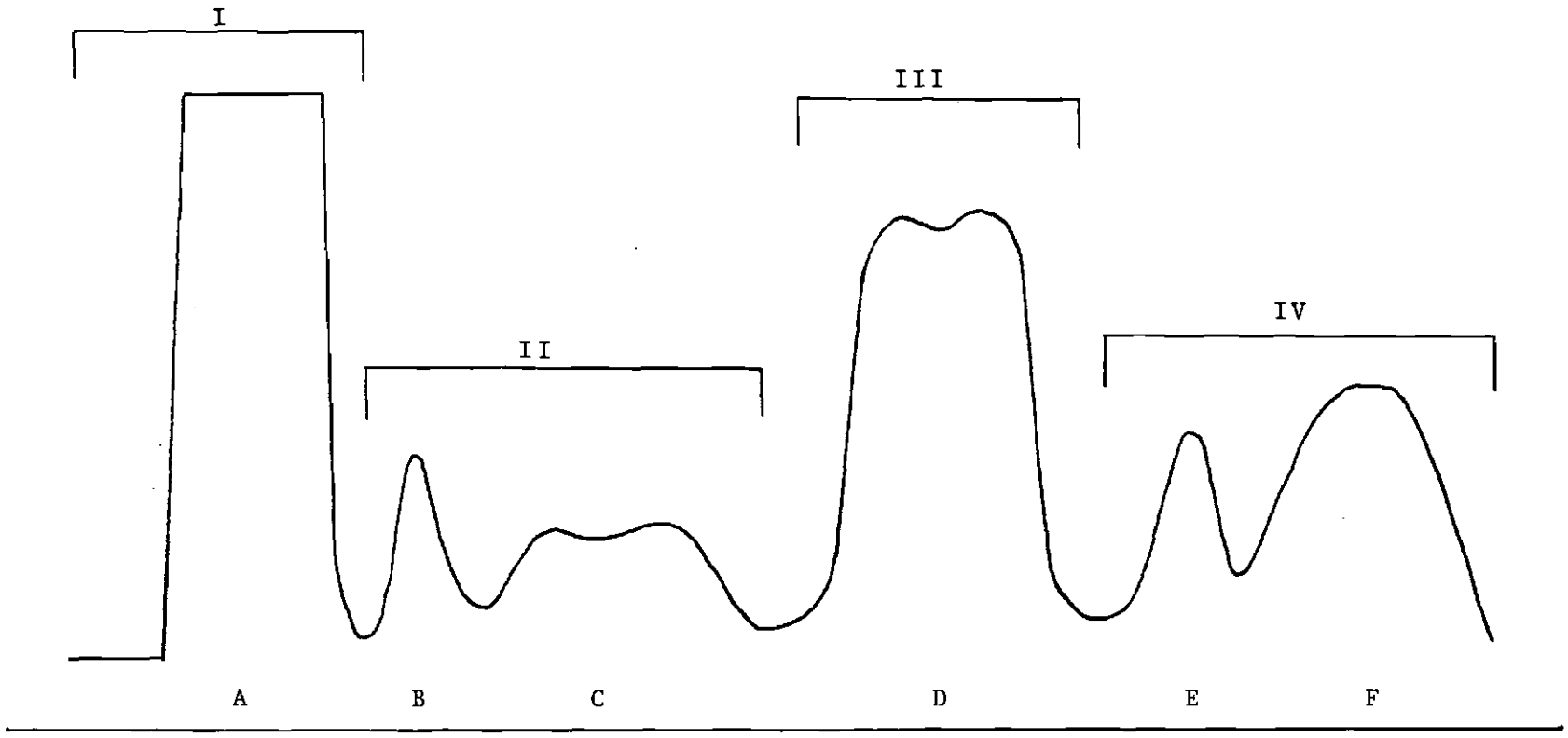


Figure 9. Elution Profile of a DE-52 Chromatography of a 24-60% Ammonium Sulfate Fraction. Absorbance at 280 nm. I: 0.02M Tris buffer wash. II: 0.1M Tris buffer wash. III: 0.1M KCl/.1M Tris wash. IV: 0.1M KCl-0.5M KCl/0.1M Tris gradient.

the elution of reductase occurs in the 0.1M Tris wash. The 0.1M KCl/0.1M Tris wash resulted in a dark yellow effluent with spectral properties including a sharp absorbance at 410 nm and low absorbances at 370 nm and 450 nm. The first effluent of the salt gradient was reddish orange with absorbances at 370 nm and 490 nm, properties characteristic of rubredoxin. The second peak from the salt gradient was colorless and had no characteristic spectral features.

The proteins eluted separately, designated A, B, C, D, E, F, Figure 9, were assayed for epoxidation activity in combination and/or in the presence of purified enzymes. The results of these assays are given in Table 2, and indicate that ion exchange chromatography on DEAE cellulose of a 24-60% ammonium sulfate fraction provides for the separation and partial purification of reductase and rubredoxin. On the basis of these assays and spectral properties, peaks E and C represent rubredoxin and reductase respectively, each free of other protein components of the P. oleovorans system. Peak D also contains reductase but the low absorbances at 370 nm and 450 nm indicate that the sample is rather dilute in reductase. Peak A, which contains a high concentration of proteins, also apparently contains some "epoxidase" and reductase since activity is observed with rubredoxin addition. Additional chromatography of Peak A on either a second DE-52 or on a

Table 2. Epoxidation Assays of Samples Eluted from a DEAE Chromatography of a 24-60% Ammonium Sulfate Fraction

Components in Reaction Mixture	7,8-epoxy-1-octene (mg/ml)
A + B + C + D + E + F	.4
Exclusion of A	0
Exclusion of B	.4
Exclusion of C	∿.1*
Exclusion of D	.3
Exclusion of E	0
Exclusion of F	.4
A + E	∿.1*
A + Rubredoxin	∿.1*
A + C + E	.4
A + E + Reductase	.4
E + Reductase	0

The proteins eluted separately on a DEAE chromatography of a 24-60% ammonium sulfate fraction were pooled and designated alphabetically (Fig. 9), then assayed individually, in combination, and in the presence of purified enzymes for epoxidation activity.

*These values are rough approximations.

Biogel A 0.5M column could not provide active preparations of reductase-free "epoxidase".

Biogel A-0.5M, 50M, Chromatography

An ammonium sulfate fraction of a 23-36% saturation was found to catalyze the epoxidation of octadiene in the presence of rubredoxin, NADH and lipid. This fraction, rich in both "epoxidase" and reductase, was applied to Biogel A chromatography. The elution profiles of both Biogel A-0.5M (10,000-500,000 Daltons) and Biogel A-50M (100,000-50,000,000 Daltons) are shown in Figure 10. The portion of the effluent, designated in Figure 10, catalyzes product formation, ∿0.1 mg/ml, only in the presence of rubredoxin and reductase, indicating that active preparations of partially purified "epoxidase" are obtained. The effluent outside the range designated requires the addition of rubredoxin alone for epoxidation activity. Although product yields are low, Biogel A chromatography does provide for the partial purification of the "epoxidase" component.

Factors Influencing Epoxidations using Partially Purified Enzyme Preparations

With the epoxidation reactions involving partially purified enzyme preparations or ammonium sulfate fractions, the addition of NADH is an absolute requirement for activity. NAD^+ cannot substitute for NADH. With all reactions involving purified enzymes, lipid was included at a

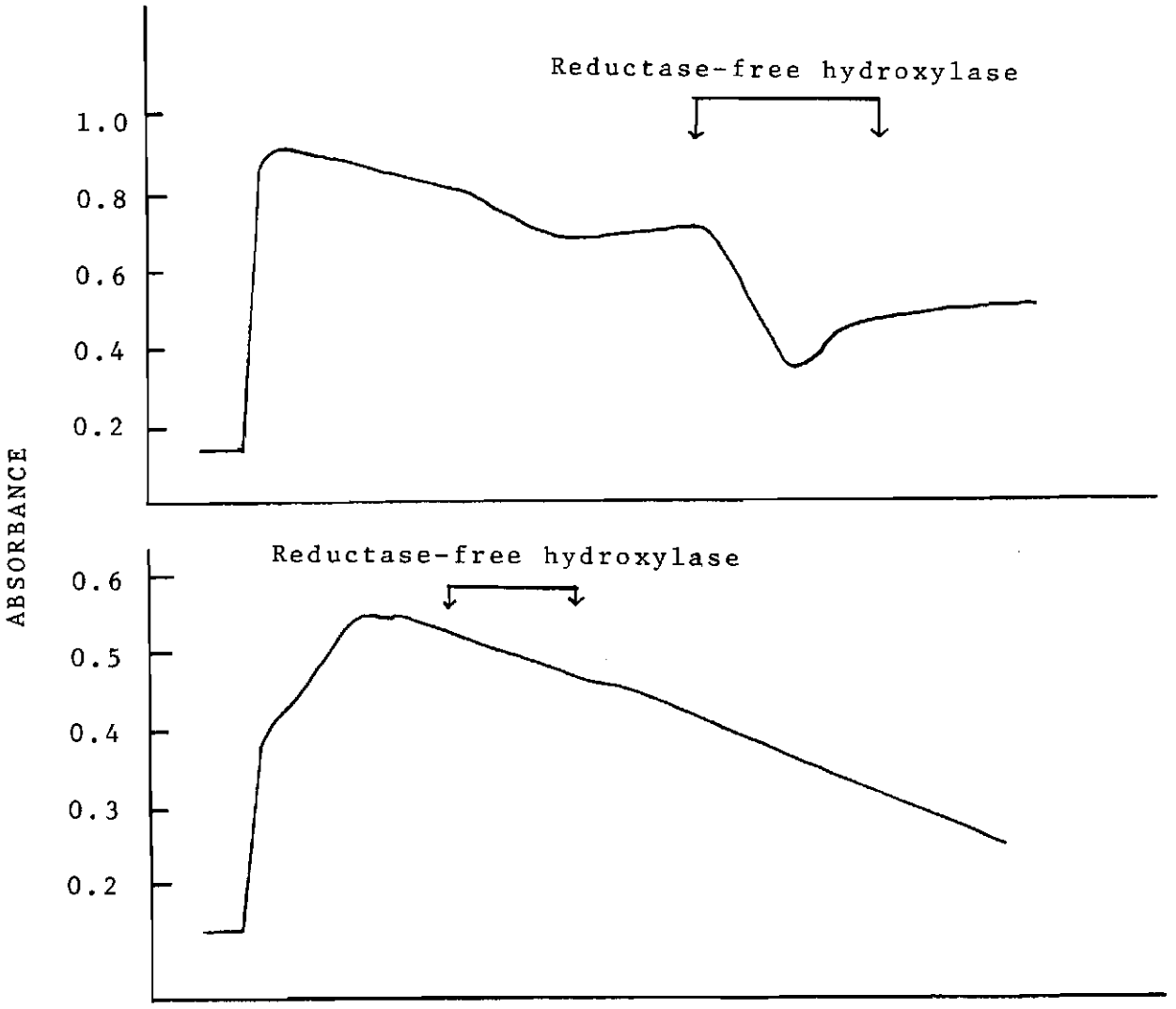


Figure 10. Top: Elution Profile of a Biogel A-50M Chromatography of a 23-36% Ammonium Sulfate Fraction. Bottom: Elution Profile of a Biogel A-0.5M Chromatography of a 23-36% Ammonium Sulfate Fraction

concentration of 1 mg/ml. The exclusion of lipid results in an almost complete loss of activity. The phospholipid requirement may account in part for the instability of the enzyme preparation. A considerable decrease in activity is often observed with the enzyme preparations upon prolonged storage at 0°C. It has previously been reported that lipid facilitates electron transfer in a cytochrome P-450-containing system,[62] however, such an effect in the P. oleovorans system remains to be established.

A ferrous iron requirement has been established for the P. oleovorans system.[12] The fact that ferrous iron addition does not provide any significant stimulatory effect indicates that no appreciable depletion of iron occurs during purification.

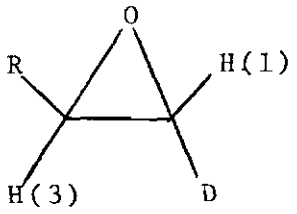
Partially Relaxed Proton Fourier Transform NMR Spectra of the Enzymatic Epoxidation Product of trans, trans-1,8-dideuterio-1,7-octadiene

The epoxidation of the deuterated substrate was carried out using a resting cell suspension under optimal conditions, and the product was isolated and purified as described in the experimental section. Approximately, 450 mg of the final purified product was obtained.

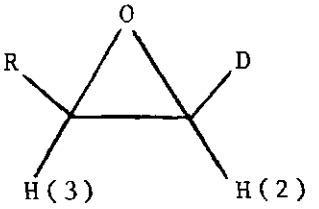
Partially relaxed proton FT NMR spectra of a solution of the enzymatic product mixture in CCl_4 were obtained at 99.5 MHz using a 180°-τ-90° pulse sequence as

described. An equilibrium spectrum of the enzymatic product mixture is given in Figure 11, which includes a structural formula of 7,8-epoxy-1-octene with the numbering of the relevant protons. Coupling constants and chemical shifts are given in the figure legend, with the lowest field line of the H (1) multiplet assigned zero frequency. Spectral features have been previously assigned.[22]

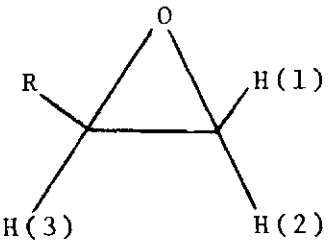
The partially relaxed spectra show that the NMR signals in the epoxide region are due to the trans deuterated epoxide I, the cis deuterated epoxide II, and the fully protonated epoxide III, and the NMR signals in the olefin region are due to the trans deuterated olefin IV



I



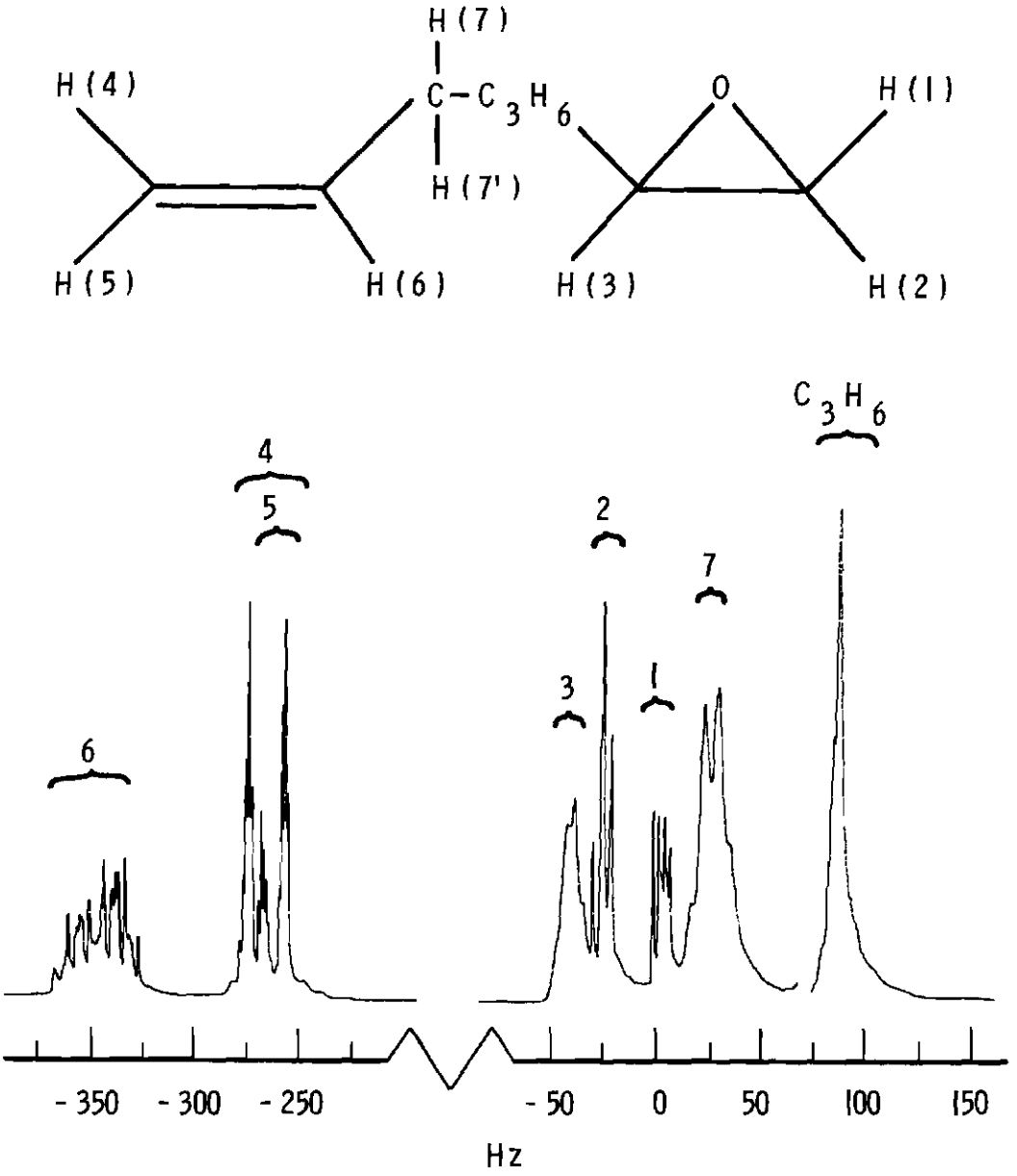
II

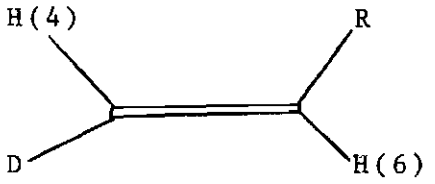


III

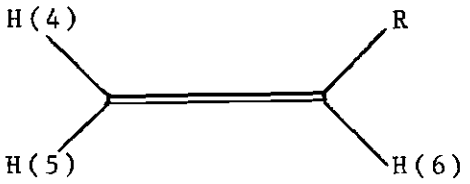
and the fully protonated olefin V.[63]

Figure 11. Top: Structural formula of 7,8-epoxy-1-octene with numbering system of protons. Bottom: Proton FT NMR spectrum of enzymatic product mixture in CCl_4 at 99.5 MHz and 28°C, after 10 accumulations with a pulse repetition time between pulse sequences of 100 sec. The C_3H_6 signal has a vertical reduction of 4 with respect to the remainder of the spectrum. The NMR spectral parameters are: J_{12} = 5.4 Hz, J_{13} = 2.4 Hz, J_{23} = 3.9 Hz, J_{45} = -2.2 Hz, J_{46} = 17.1 Hz, J_{47} = 1.4 Hz, J_{56} = 10.0 Hz, J_{57} = 1.2 Hz, J_{67} = 6.6 Hz, ν_{01} = 4.0 Hz, ν_{02} = -24.0 Hz, ν_{03} = -40 Hz, ν_{04} = -263.9 Hz, ν_{05} = -259.1 Hz, ν_{06} = -342.6 Hz, ν_{07} = 27 Hz, where the lowest field line of the H(1) multiplet is zero frequency.


H(7)
H(4)
C-C3H6
O
H(1)
H(7')
H(5)
H(6)
H(3)
H(2)
C3H6
4
5
2
7
6
3
1
-350
-300
-250
-50
0
50
100
150
Hz



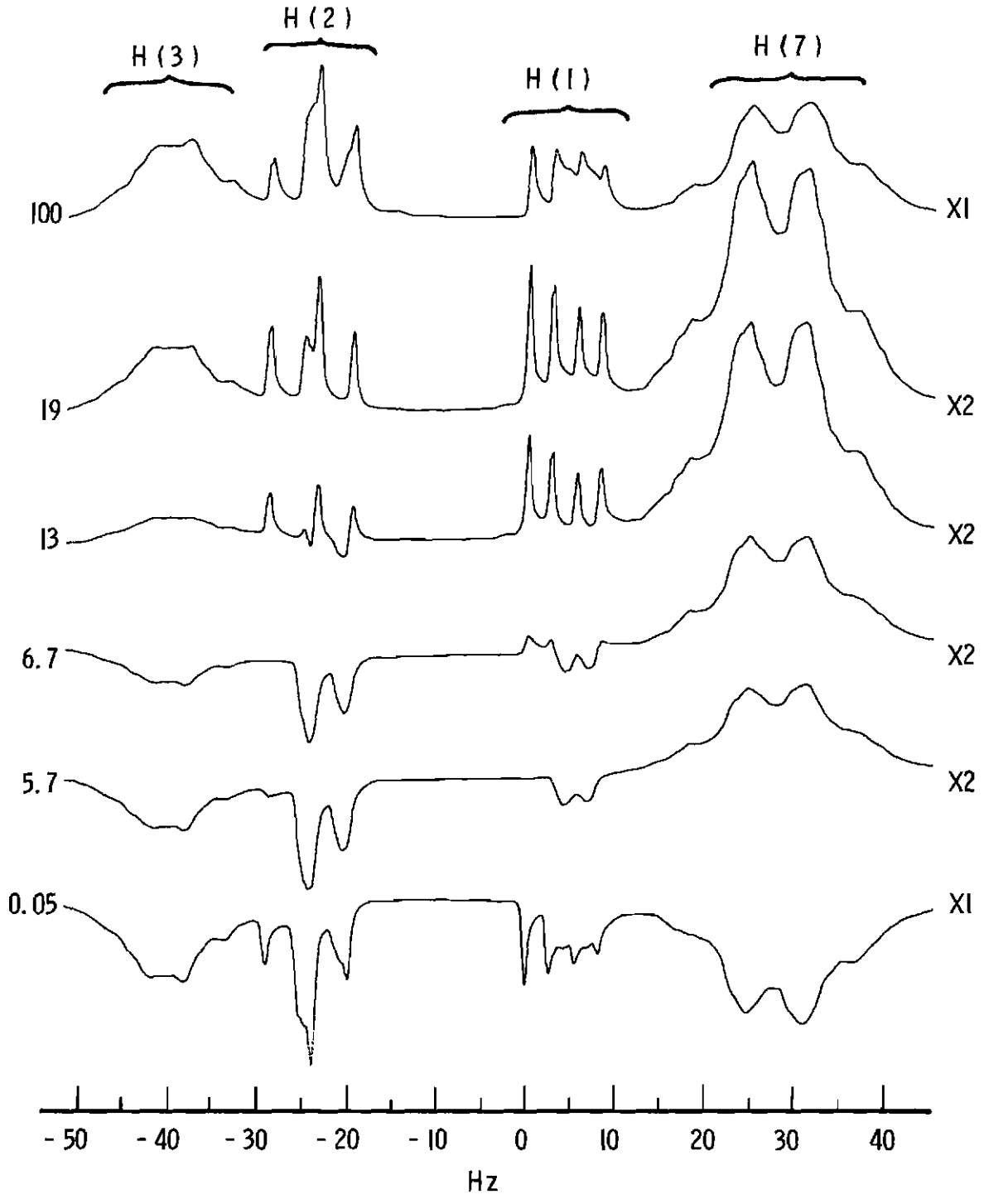
IV



V

Partially relaxed spectra of the epoxide region are shown in Figure 12, with τ increasing from bottom to top. The bottom trace with τ = 0.05 sec. is an inverted spectrum. At τ = 5.7 sec., a broad inverted doublet of splitting 2.4 Hz is isolated in the H(1) region. This signal is due to H(1) of I, and is the X proton of an approximate AX pattern.[64,65] The broadening is due to an unresolved H-D splitting of 0.7 Hz. At τ = 6.7 sec., another broad inverted doublet of 3.7 Hz splitting is isolated in the H(2) region. This signal is due to H(2) of II, and is the B portion of an AB quartet. The H(2) doublet is considerably more intense than the H(1) doublet. From the τ = 5.7 sec. and τ = 6.7 spectra, we see that both the trans and cis deuterated epoxides were produced but that the cis isomer was the dominant product. Therefore, we conclude that the enzymatic epoxidation reaction has occurred predominantly with inversion of the original double bond configuration. The signals due to H(1) and

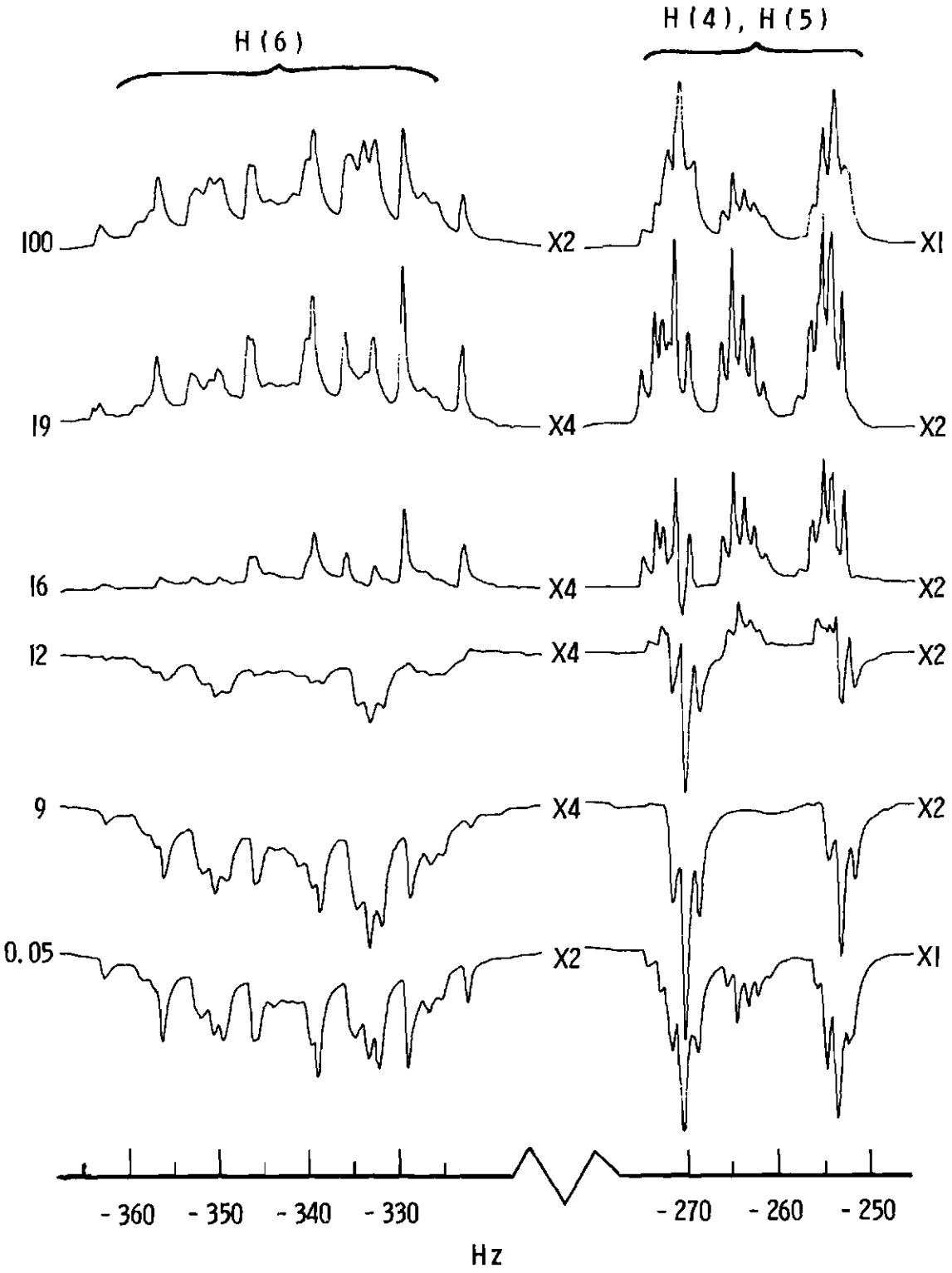
Figure 12. Partially relaxed proton FT NMR spectra of the epoxide region of the enzymatic product mixture in CCl_4 at 99.5 MHz and 28°C. The number to the left of each spectrum is τ in seconds. Each spectrum is the result of 16 accumulations with a repetition time of 100 sec. The relative vertical expansion factors are given to the right of each spectrum.


H(3)
H(2)
H(1)
H(7)
100
19
13
6.7
5.7
0.05
X1
X2
X2
X2
X2
X1
-50
-40
-30
-20
-10
0
10
20
30
40
Hz

H(2) of the fully protonated epoxide III are isolated at τ = 13 sec. and τ = 19 sec., respectively, and form the BX portion of an approximate ABX pattern. From these spectra we calculate J_{13} = 2.4 Hz, J_{23} = 3.9 Hz, and J_{12} = 5.4 Hz in good agreement with coupling constants reported for other epoxides.[66,67] The basis for the epoxide spectral assignment is the observation that trans coupling constants in trans disubstituted epoxides have a characteristic value of about 2 Hz.[42] It will be seen below that our relaxation time measurements and the results obtained with the peracid epoxidation product provide additional support for the assignment.

Partially relaxed spectra of the olefin region are shown in Figure 13. At τ = 9 sec., an inverted doublet of triplets is isolated in the H(4), H(5) region with a doublet splitting of 17.1 Hz and a triplet splitting of 1.4 Hz. This signal is assigned to H(4) of IV, and is the B portion of an ABX_2 pattern where A, B, X correspond to H(6), H(4), H(7), respectively. The H(4) and H(5) signals of V appear to have both relaxed to zero intensity at τ = 9 sec. Actually, a vertical expansion indicated that the H(4) signal of V relaxed somewhat earlier at about τ = 8.5 sec. The partially relaxed spectrum at τ = 9 sec. is fully consistent with our contention that no isomerization of the trans deuterated olefin functionality has occurred. If any cis deuterated olefin had been present, we would

Figure 13. Partially relaxed proton FT NMR spectra of the olefin region of the enzymatic product mixture in CCl_4 at 99.5 MHz and 28°C. The number to the left of each spectrum is τ in seconds. Each spectrum is the result of 16 accumulations with a repetition time of 100 sec. The relative vertical expansion factors for the H(6) and H(4), H(5) regions of the spectra are different and are indicated to the right of the respective regions on each trace. Zero frequency is assigned to low field line of the H(1) multiplet (see Figure 12).


H(6)
H(4), H(5)
100
X2
X1
19
X4
X2
16
X4
X2
12
X4
X2
9
X4
X2
0.05
X2
X1
- 360
- 350
- 340
- 330
- 270
- 260
- 250
Hz

have seen substantial inverted signals at about -265 Hz. The H(4), H(5) signals of V are isolated at τ = 19 sec., giving the MN portion of an approximate $AMNX_2$ pattern. At τ = 12 sec., an inverted pattern is isolated in the H(6) region. This is due to H(5) of IV, and is the A portion of an ABX_2Y spectrum with an AB splitting of 17.1 Hz due to H(4), AX_2 triplet splitting of 6.6 Hz due to the H(7) protons, and an AY triplet splitting of 1.5 Hz due to the deuterium. At τ = 16 sec., the signal due to H(6) of structure V is isolated, and is the A portion of an approximate $AMNX_2$ pattern, with an AX_2 triplet splitting of 6.6 Hz with the H(7) protons. As expected, the equilibrium spectrum can be seen to be a superposition of the various patterns isolated in the partially relaxed spectra.

Quantitative Analysis

In order to quantitatively analyze the partially relaxed spectra, we made the simplest relaxation assumption, namely that each multiplet A relaxes exponentially with an effective spin lattice relaxation time T_{1A}. This assumption cannot be strictly correct for our sample because the proton relaxation is dominated by the intramolecular dipolar mechanism. However, effective relaxation times as defined above have proved useful in studying molecular motion[68,69] and they adequately describe the partially relaxed spectra of our sample. The validity of this assumption for our

samples was checked in the following ways. In the first place, the intensities of the epoxide multiplets (which have been estimated from the intensities of the low field exposed lines arising from the fully protonated species) were plotted logarithmically vs. time and in every case these plots were linear. Furthermore, the effective relaxation times obtained from these plots were in good agreement with the values computed from the experimentally measured null times. In addition, the calculated I(0) values listed in Table 3 were used to predict the total intensities of the fully inverted epoxide multiplets, and these agreed within about 5-10% with the experimentally measured intensities.

With this assumption, the multiplet A, after inversion by the 180° pulse relaxes to zero intensity at the null time τ_A, where

$$\tau_A = T_{1A} \ln(1+\alpha) \qquad (1)$$

In Equation (1), α, the fractional inversion, is given by

$$\alpha = -I_A(0)/I_A(\infty)$$

where $I_A(0)$ is the intensity of the inverted signal (i.e., $\tau \ll T_{1A}$), and $I_A(\infty)$ is the intensity of the equilibrium

signal (i.e., $\tau >> T_{1A}$). Therefore, if A and B are overlapping multiplets, signal A is isolated at $\tau = \tau_B$, and B is isolated at $\tau = \tau_A$. From a knowledge of τ_A, τ_B, $I_A(\tau_B)$, and $I_B(\tau_A)$, we can compute the intensity of A in the inverted spectrum, $I_A(0)$, from

$$I_A(0) = \frac{\alpha I_A(\tau_B)}{(1+\alpha)\ \exp\left(\frac{-\tau_B}{\tau_A}\ \ln(1+\alpha)\right) - 1} \qquad (2)$$

and T_{1A} from Equation (1), with analogous formulas for $I_B(0)$ and T_{1B}.

The observation times, null times, and relative integrated intensities of the isolated multiplets of Figures 12 and 13 are recorded in Table 3. Each intensity is an average from 3 spectra. The fractional inversion was determined from the olefin region to be 0.94 ± 0.02. Using this value of α, T_{1A} and $I_A(0)$ were computed for each multiplet from Equations (1) and (2). The values of T_{1A} and $I_A(0)$ are given in Table 3. From the $I_A(0)$ values for H(1) of I and H(2) of II, it is evident that 70% of the deuterated epoxide is the cis isomer.

We can look for deuterium isotope effects by comparing the deuterium content of the epoxide portion

Table 3. Null Times, Relaxation Times, and Intensities of Epoxide and Olefin Multiplets

Multiplet	Observation Time (sec)	Null Time (sec)	T_1[a] (sec)	I[b] at observe time	I(0)[d]	Predicted T_1 (sec)
H(1) I	5.7	13	20	-126 (2)	-265	18
H(2) II	6.7	19	29	-349 (16)	-616	-
H(1) III	13	5.7	8.6	398 (27)	-649	8
H(2) III	19	6.7	10	437 (17)	-580	9
H(3) I, II, III	-	10	15	-	-	11
H(4) IV	9	19	29	-457 (19)	-1039	
H(6) IV	12	16	24	-	-	
H(4) V	19	8.5	13	753 (56)[c]	-1298[c]	
H(5) V	19	9	14			
H(6) V	16	12	18	-	-	

a. Computed from Equation (1) with $\alpha = 0.94$.
b. Average of 3 measurements, average deviation is given in parenthesis.
c. Sum of intensities for H(4) and H(5).
d. Computed from Equation (2) with $\alpha = 0.94$.

with that of the olefin portion of the epoxide product. From the $I_A(0)$ values for H(1) and H(2) of I, II and III, we compute that 59% of epoxidation product was deuterated. From the $I_A(0)$ values of H(4) of IV and H(4), H(5) of V, we calculate that 62% of the olefin substrate was deuterated. The percent deuteration in the olefin was also computed from the equilibrium spectrum by comparing the integrated intensity of the H(5) region with that of the H(4), H(5) region. These measurements gave 62% in good agreement with the partially relaxed spectra result. Therefore, within experimental error, a significant secondary deuterium isotope effect would not be detected by this technique.

Partially relaxed spectra also were obtained for a second batch of enzymatic epoxidation product. The NMR spectra showed that the substrate used for this batch contained 29 mole per cent of the trans deuterated and 71 mole per cent of the fully protonated 1,7-octadiene. This sample provided a test of the sensitivity of the partially relaxed technique because the fraction of deuterated material was low and only 50 mg of epoxidation product were available. The experimental results were virtually identical to those obtained with the first batch, with 71% of the enzymatic product being the cis deuterated epoxide.

Relaxation Analysis

The relaxation times for the gem protons can be used to compute T_{ij}, the contribution to relaxation from pairwise dipolar interactions between H(i) and H(j). T_{ij}, in turn, provides the value of the reorientational correlation time τ_R from the well known formula[70]

$$T_{ij}^{-1} = \frac{3}{2} \gamma^4 h^2 r_{ij}^{-6} \tau_R \qquad (3)$$

where γ is the proton magnetogyro ratio, and r_{ij} is the distance between H(i) and H(j). If it is assumed that the geometry of the olefin and epoxide portions of the product are the same as those of propylene and propylene oxide, respectively,[71] then the dimensions of the H(1), H(2), H(3), and the H(4), H(5), H(6) triangles are identical, and $r_{12} = r_{45} = 1.84\text{Å}$. We compute $\tau_R = 3.02 \times 10^{-12}$ sec. for the epoxide group, using the H(1) relaxation times in Table 1 and Equation (3). As a check, we can also compute the epoxide τ_R from the H(2) relaxation times, and again we obtain 3.02×10^{-12} sec. From the H(4) relaxation times we calculate $\tau_R = 1.92 \times 10^{-12}$ sec. for the olefin group. Therefore, the olefin portion of the molecule reorients considerably faster than the epoxide portion.

If we assume that the difference in relaxation rates of corresponding epoxide and olefin protons is due

to the difference in correlation times, we can predict each epoxide relaxation time by multiplying the corresponding olefin relaxation time by 1.92/3.02. The epoxide relaxation times predicted in this manner are given in the last column of Table 3. The agreement is satisfactory, and provides strong support for the spectral assignment of the H(1) and H(2) epoxide protons.

It is interesting to note that H(1) relaxes faster than H(2) and that H(4) relaxes faster than H(5), even though H(2) and H(5) are both cis to a proton on the adjacent carbon. The cis interaction, in fact, allowed us to successfully isolate the H(6) signals of IV and V. The faster relaxation times of H(1) and H(4) can be rationalized on the basis that they are closer to the numerous protons of the methylene chains of the respective compounds than are H(2) or H(5).

Analysis of the Peracid Epoxidation Product

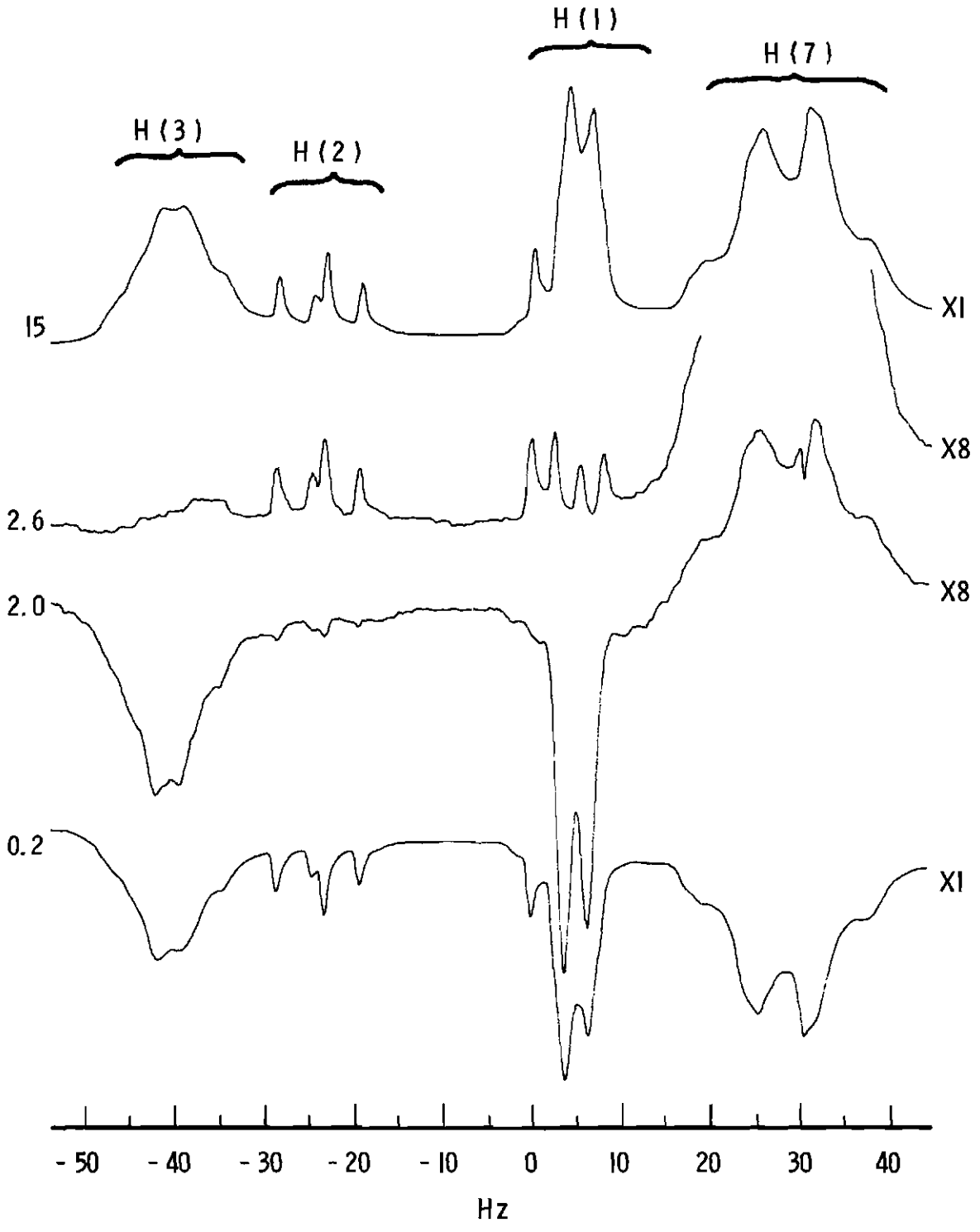
In order to compare directly the enzymatic and peracid epoxidation processes, the monoepoxidation product of trans, trans-1,8-dideuterio-1,7-octadiene was synthesized using m-chloroperbenzoic acid. NMR analysis showed that the starting material used in this synthesis consisted of 78% of the deuterated and 22% of the fully protonated 1,7-octadiene. Partially relaxed proton FT NMR spectra of a non-degassed sample of the peracid epoxidation product

in CCl_4 were obtained. The accumulation conditions were the same as for the previous samples, except that because of the short relaxation times of the sample, a recycle time between pulse sequences of 15 sec. was used. The epoxide region is shown in Figure 14. The relevant multiplets are cleanly isolated even though the dominant proton relaxation mechanism in this sample is intermolecular dipolar interaction with dissolved oxygen. The signal to noise of the isolated multiplets is lower than that of Figure 13 because the difference in null times is small.

The H(1) doublet of I is isolated at τ = 2.0 sec. The quartet due to H(1) of the fully protonated epoxide III is isolated at τ = 2.6 sec. There is no indication of an H(2) doublet arising from a cis-deuterated epoxide. Therefore, by comparison of Figures 12 and 14, we conclude, without making any assumptions whatsoever regarding spectral assignments, that the enzymatic epoxidation reaction occurs predominantly with inversion of the original double bond configuration, while peracid epoxidation occurs, as expected, with complete retention of configuration.

The deuterium content of the epoxide and olefin regions was computed in the same manner as discussed for the enzymatic product and the results showed that 80% of the epoxidation product and 78% of the olefin reactant were deuterated. Therefore, within experimental error, no significant secondary deuterium isotope effect could be

Figure 14. Partially relaxed proton FT NMR spectra of the epoxide region of the peracid product mixture in CCl_4 at 99.5 MHz and 28°C. The sample was not degassed. The number to the left of each spectrum is τ in seconds. Each spectrum is the result of 16 accumulations with a repetition time of 15 seconds. The relative vertical expansion factors are given to the right of each spectrum.


H(1)
H(7)
H(3)
H(2)
15
XI
X8
2.6
X8
2.0
0.2
XI
-50
-40
-30
-20
-10
0
10
20
30
40
Hz

detected for the peracid epoxidation reaction using this technique.

Control Experiments for Epoxide Product Isomerization

Conceptually, it might be postulated that non-enzymatic isomerization processes occur under the conditions of our experiments which could complicate or invalidate our results. It is clear from the completely trans configuration of the peracid-generated epoxide that such processes do not occur during the product isolation steps (preparative gas chromatography) or NMR analysis, and the completely trans configuration of the olefin portion in enzymatically-generated 7,8-epoxy-1-octene clearly establishes that no isomerization of the olefin functionality occurs during the enzymatic reaction or subsequent steps. As far as possible isomerization of the newly-enzymatically-generated epoxide functionality in the resting cell suspension is concerned, it could be argued that it is extremely unlikely such a process would account for the net inversion of configuration we observe, and at most, might be responsible for our seeing 70% cis rather than, say, 90-100% cis configuration in the enzymatically produced epoxide functionality. Even if this were the case, our major mechanistic conclusion that a concerted mechanism of oxygen addition is untenable would be even stronger. However, despite these arguments we proceeded

to run the following control experiments in order to unequivocally demonstrate that isomerization of the epoxide functionality does not occur under the conditions of our experiments.

(1) The peracid epoxidation product from trans, trans-1,8-dideuterio-1,7-octadiene, which we have demonstrated above (Figure 13) to contain only the trans deuterated epoxide functionality, was incubated with a resting cell suspension of P. oleovorans under conditions identical to those used for enzymatic epoxidation of deuterated octadiene. Extraction of the reaction mixture with ether followed by preparative gas chromatography allowed reisolation of the monoepoxide, which was then analyzed by NMR as described above. The reisolated compound exhibited no isomerization of either the epoxide or olefin functionalities, the NMR spectrum after incubation being identical to that of the starting material.

(2) Starting from pure samples of each of the isomeric internal olefins cis and trans-3-hexene, the compounds trans-3,4-epoxyhexane and cis-3,4-epoxyhexane were synthesized by m-chloroperbenzoic acid epoxidation. A series of NMR spectra on various mixtures of the two epoxides was obtained and it was found that the presence of as little as 5% of the cis epoxide in the presence of trans-3,4-epoxyhexane could easily be detected. A sample of the trans epoxide was then incubated with a resting

cell suspension of P. oleovorans under conditions identical to those previously used for epoxidation of deuterated octadiene, and the compound was then reisolated and purified by preparative gas chromatography. Once again, no isomerization of the trans epoxide was detected by careful NMR analysis.

Optical Rotation of Epoxides

Figure 15 shows the ORD curves obtained with neat samples of the various enzymatically and chemically produced epoxides. All samples were checked carefully for purity and the only contamination was 1-2% of racemic monoepoxide in the enzymatically produced diepoxide sample. (Control experiments were carried out to establish that the presence of this small amount of racemic monoepoxide had a negligible effect on the reported specific rotation of the diepoxide.) As reported previously,[24] neat 7,8-epoxy-1-octene produced enzymatically from octadiene exhibits a positive rotation while that produced from the peracid epoxidation of octadiene is racemic. The data presented in Figure 15, establishes that enzymatically produced 1,2-7,8-diepoxy octane from octadiene also exhibits a positive rotation.

The specific rotations for the enzymatically produced epoxides are $[\alpha]_D^{25} = +13.2$ and $[\alpha]_D^{25} = +20.8$ for 7,8-epoxy-1-octene and 1,2-7,8-diepoxyoctane respectively. However, it is apparent that the magnitude of the positive

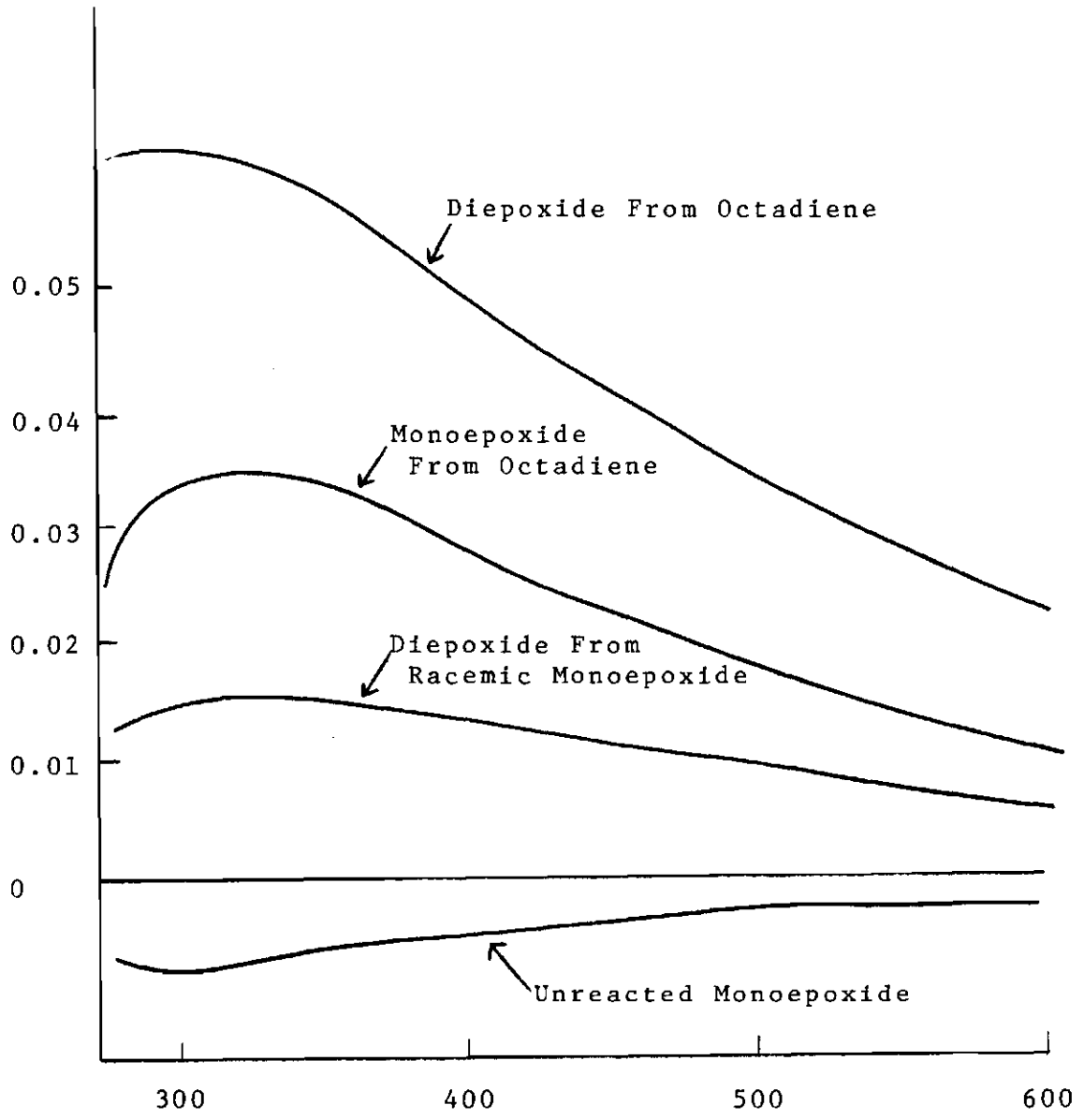


Figure 15. ORD Curves for Various Enzymatically Produced Mono- and Diepoxide Products. The spectra were obtained using neat samples in a 0.1 mm pathlength cell.

rotation exhibited by neat samples of enzymatically produced 1,2-7,8-diepoxyoctane is strikingly dependent upon whether the starting substrate is octadiene or racemic 7,8-epoxy-1-octene. In the latter case, the diepoxide has a positive rotation with $[\alpha]_D^{25}$ = + 5.38 and the unreacted monoepoxide exhibits a negative rotation with $[\alpha]_D^{25}$ = -2.9.

Enantiomeric Composition of Epoxides

The optical purities of the various epoxides were determined by NMR using the chiral shift reagent Tris [3-(trifluoromethylhydroxymethylene)-3-camphorato]europium III. Figure 16 shows the effect of the concentration of europium shift reagent on the chemical shift of H_1, H_2 and H_3, and upon the separation of the isomeric multiplets associated with H_2 and H_3 for 1,2-7,8-diepoxyoctane. Figure 17 shows a similar effect for the epoxy and olefinic protons of 7,8-epoxy-1-octene. As shown in Figure 17, H_2 and H_3 are split, corresponding to the R and S isomers, with the splitting of H_2 being the most pronounced. H_2 is split into two well defined triplets and H_3 into two broad multiplets. For H_1, H_2 and H_3, addition of the shift reagent results in a considerable downfield shift, whereas the methylene protons are shifted only slightly downfield.

With the addition of 0.1188 moles of shift reagent to 0.395 mmoles of 1,2-7,8-diepoxyoctane, H_1 is shifted from δ2.33 to δ3.55, H_2(S) from δ2.56 to δ3.90, H_2(R) from δ2.56 to δ4.2, and H_3 from δ2.75 to δ4.60. The separation

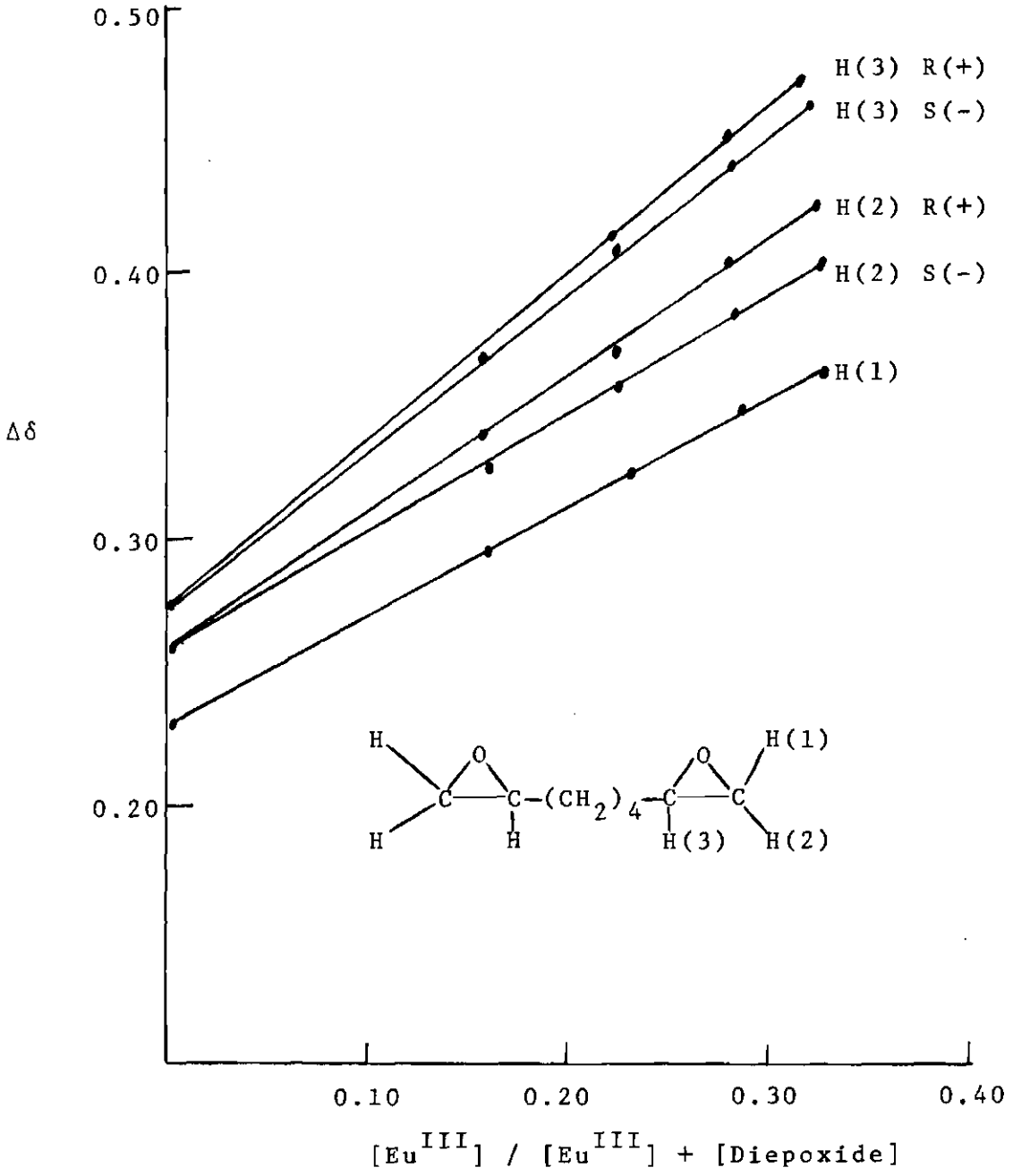


Figure 16. Effect of $[Eu^{III}]$ on the Change in Chemical Shift and on the Separation of the Isomeric Multiplets Associated with H(3) and H(2) of 1,2-7,8-diepoxyoctane

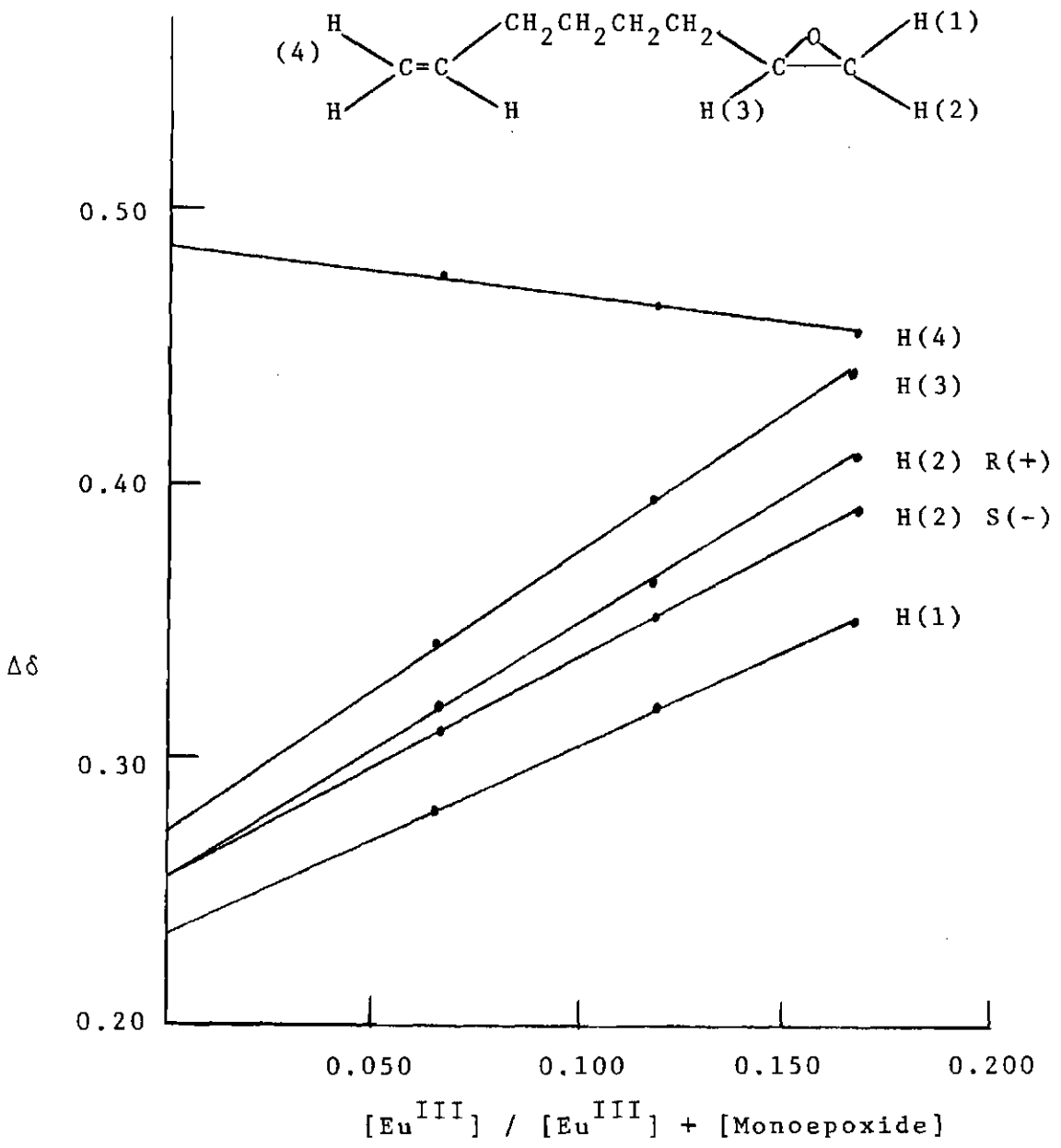


Figure 17. Effect of $[Eu^{III}]$ on the Separation of the Isomeric Triplets Associated with H(2) and the Change in Chemical Shift of Several Protons of 7,8-epoxy-1-octene

of the two isomeric triplets associated with H_2 was sufficient for quantitative determinations. The downfield triplet arises from the isomer with the positive rotation which has the R configuration.[24,72,73]

For 7,8-epoxy-1-octene, Figure 17, the addition of 0.024 mmoles of shift reagent to 0.258 mmoles of epoxide is sufficient for separating the isomeric triplets. Greater amounts of shift reagent results in an overlapping of the triplets with the peaks associated with the olefinic protons which were shifted slightly upfield.

For the monoepoxide and diepoxide samples, a quantitative determination of the R and S isomers is summarized in Table 4. Clearly, the peracid epoxidation of octadiene gives both racemic mono- and diepoxides whereas the enzymatic epoxidation of octadiene proceeds with a high degree of stereoselectivity to give preferentially the R-monoepoxide and the R,R-diepoxide.

Conceptually, several limiting possibilities can be envisioned for the stereochemical course of the enzymatic epoxidation of (R,S)-7,8-epoxy-1-octene. On the one hand, in view of the preferential formation of (R) monoepoxide from octadiene, it might be expected that enzymatic oxygen insertion always proceeds virtually exclusively from the si-si face of the reactive double bond, irrespective of the configuration of a preformed asymmetric center at the other end. Thus, racemic monoepoxide would give rise to a mixture

Table 4. Enantiomeric Composition of Epoxides

Compound	NMR Determination[a] % R Sites[b]	% S Sites[b]
Peracid Epoxidation of 1,7-octadiene		
7,8-epoxy-1-octene	50	50
1,2-7,8-diepoxy octane	50	50
Enzymatic Epoxidation of 1,7-octadiene		
7,8-epoxy-1-octene	92	8
1,2-7,8-diepoxy octane	83	17
Enzymatic Epoxidation of Racemic 7,8-epoxy-1-octene		
1,2-7,8-diepoxy octane	59	41
Unreacted 7,8-epoxy-1-octene	47	53

a. Optical purity determinations determined using the Europium shift reagent as described in text.

b. All values are averages of at least three determinations. Average deviations never exceeded about 2%.

of (R,R) and (S,R) diepoxide molecules, and the enantiomeric excess of R sites should approach 50%. A second limiting possibility is that the configuration of a preformed asymmetric center somehow alters the course of the enzymatic oxygen insertion process so that, for example, (R) monoepoxide might give rise to only (R,R) diepoxide and (S) monoepoxide to only (S,S) diepoxide, thus resulting in a racemic diepoxide from a racemic monoepoxide. Obviously, any number of intermediate possibilities with (R) or (S) monoepoxide functionalities exerting various degrees of influence on the stereochemical course of the enzymatic diepoxidation process may be envisioned. In addition, the monoepoxide enantiomers may be oxygenated at different rates with the limiting cases being that either one or the other is essentially unreactive in this process. Analysis of the data presented in Table 4 reveals that they are consistent with the conclusion that diepoxidation of the S-monoepoxide produces predominantly (S,S) diepoxide and diepoxidation of R-monoepoxide produces predominantly (R,R) diepoxide, the stereoselectivity in both cases being about 90%. Thus, starting with A moles each of R- and S- monoepoxides in the racemic starting material, reaction of X moles of R to produce 90% (R,R) and 10% (R,S) diepoxide would result in an excess of 1.8(X-Y) R sites in the diepoxide product and a corresponding excess of (Y-X) S sites in the unreacted monoepoxide. The observed enantiomeric excesses of approximately 20% R sites

in the diepoxide product and approximately 10% S sites in the unreacted monoepoxide is thus consistent with this scheme. However, from the data at hand, other more complex schemes cannot be ruled out. For example, a scheme which postulates the generation of (R,R) diepoxide from R-monoepoxide and random generation of (S,R) and (S,S) diepoxides from S-monoepoxides is also consistent with our results if $Y < X$. A final determination on this point must await the development of new methods capable of generating the S-monoepoxide in high optical yield so that it can be used as a substrate in the enzymatic reaction and the stereochemistry of the product determined directly. In any case, it is clear that the opposite ends of a straight chain compound do not operate independently in this enzymatic system. May and coworkers have previously noted the unusually critical role played by the mode of substrate binding in moderating the reactivity of this enzymatic system,[23,27-29] and our results with the racemic monoepoxide undoubtedly reflect such factors.

Incubation of Allylic Alcohols with P. oleovorans

A series of allylic alcohols were investigated as possible substrates for the P. oleovorans epoxidation system to determine the effect of a hydroxyl substituent upon the reactivity and selectivity of the epoxidation reaction. In chemical systems, the effects of hydroxyl substituents

on epoxidation reactions have been described.[40,74,75] For example, with peracid epoxidations, allylic alcohols lower the reactivity of the double bond, whereas in transition metal catalyzed epoxidations with alkyl hydroperoxides, the hydroxyl substituent often increases the reactivity of the double bond. In both cases, a syn directive effect is observed on the stereochemistry of the reaction.

The enzymatic reactions with several allylic alcohols were carried out using cell-free preparations and the retention times compared to the products obtained in the reactions with m-chloroperbenzoic acid. In no case did the retention time of the enzymatic product correspond to the peracid product. The peracid products were confirmed to be the corresponding epoxides by NMR and mass spectral analysis. On the basis of NMR, mass spectral and retention time comparisons to authentic samples (see Experimental section), the enzymatic products were identified to be the corresponding saturated ketones. Thus, as shown in Table 5, 1-octen-3-ol is converted to 3-octanone and 2-methyl-1-hepten-3-ol is converted to 2-methyl-3-heptanone by the P. oleovorans system. The incubation of 1-penten-3-ol results in only a negligible amount of product formed and efforts in obtaining pure product for characterization using either a cell-free or resting cell suspension on larger scales proved fruitless. The incubation of 3,7-dimethyl-1,6-octadien-3-ol (linalool), did not result in any product

formation when carried out with either cell-free or resting cell preparations.

For both 1-octen-3-ol and 2-methyl-1-hepten-3-ol, minor products were detected. Although product yields were low and the minor products were not isolatible, a comparison of retention times with authentic samples indicated that the minor products formed from 1-octen-3-ol and 2-methyl-1-hepten-3-ol are apparently 3-octanol and 2-methyl-1-hepten-3-one respectively. The possible involvement of these minor products as intermediates is further indicated in that the cell free incubation of 3-octanol and 2-methyl-1-hepten-3-one both resulted in the formation of the corresponding saturated ketone. (See Table 5).

The data presented in Table 5, indicate that the presence of an allylic hydroxyl substituent prevents the epoxidation of the terminal double bond by the Pseudomonas oleovorans system. Although an optimization study was not carried out, the conversion of 1-octen-3-ol to 3-octanone approached product yields of 1.5 mg/ml, comparable to yields obtained in the cell-free epoxidation of 1,7-octadiene, and the conversion of 2-methyl-1-hepten-3-ol to 2-methyl-3-heptanone approached yields of 1 mg/ml. These results demonstrate a new and potentially synthetic feature of the P. oleovorans system.

Table 5. Identification and Quantitation of Products from Cell Free Incubations of α,β-Unsaturated Ketones and Allylic Alcohols

Substrate	Product	Yield vs. Reaction Time (μg/ml) 10h	20h	40h
1,7-octadiene	7,8-epoxy-1-octene	800	1500	1300
1-octen-3-ol	3-octanone	500	1000	1100
	3-octanol[1]	<100	200	200
1-octen-3-one	3-octanone[1]	100	200	200
3-octanol	3-octanone	800	1200	1200
2-methyl-1-hepten-3-ol	2-methyl-3-heptanone	300	500	700
	2-methyl-1-hepten-3-one[1]	100	300	400
2-methyl-1-hepten-3-one	2-methyl-3-heptanone	500	600	700
Linalool (3,7-dimethyl-1,6-octadien-3-ol)		NO REACTION		
1-penten-3-ol	Not Identified		trace	

Incubations carried out with a cell-free preparation of P. oleovorans and quantitated by gas chromatography using 1-octanol as an internal standard. Product identification based upon comparisons of NMR and mass spectral analysis to those for authentic samples.

[1]Identification based on only retention time comparisons to authentic samples.

Component Requirements for Ketonization

Since the epoxidation of the allylic alcohols by cell-free preparations does not occur, at least to any significant degree, the various components of the *P. oleovorans* system were examined to determine if the same enzyme system responsible for the epoxidation of olefins is also responsible for ketonization. As shown in Table 6, using partially purified enzyme preparations, 1-octen-3-ol was converted to 3-octanone with a product yield of about 300 µl/ml in the presence of all three protein components and NADH. This result rules out a requirement for other enzymes that exist in a crude cell free preparation. Furthermore, the data in Table 6 reveal that rubredoxin and "epoxidase" are not required in these ketonization reactions since reductase alone, in the presence of NADH, catalyzes the conversion of 1-octen-3-ol to 3-octanone with a product yield of 300 µg/ml.

When 1-octen-3-ol is incubated with General Acyl Dehydrogenase, a flavin-containing protein which binds hydrocarbon substrates, or incubated with free flavin adenine dinucleotide, no product is detected. (Table 6). These results indicate that the ketonization reaction is an enzymatic process specific for reductase, and not a general reaction catalyzed by the free or enzyme bound flavin prosthetic group.

Table 6. Effect of Deleting Components of the Enzyme Epoxidation System on the Production of 3-octanone from 1-octen-3-ol.

System	Product Formation[1] (µg/ml)
Rubredoxin, Reductase, "epoxidase"	250-300
Rubredoxin	none
"Epoxidase"	none
Reductase	300
General Acyl Dehydrogenase	none
Free FAD	none
Reductase but with NADPH instead of NADH	trace amount

[1]Analyzed quantitative gas chromatography after 3 hr. of incubating.

The reactions were carried out in the presence of NADH and oxygen as described in the Experimental section except that various components were deleted. For reactions involving General Acyl Dehydrogenase and Free Flavin Adenine Dinucleotide, 0.4 mg protein and 5.3 mmoles FAD were used.

Oxygen Requirement for Ketonization

When 1-octen-3-ol was incubated with reductase and NADH under both anaerobic (see page 36, Experimental section) and aerobic conditions, analysis by gas chromatography revealed that both reactions resulted in the formation of 3-octanone with a product yield for both about 300 µg/ml. Thus, molecular oxygen is neither a substrate nor a requirement in the reductase catalyzed ketonization reactions. In addition, oxygen monitor experiments reveal that the small endogenous rate of oxygen consumption which occurs in an air saturated solution containing reductase and NADH, decreases upon addition of 1-octen-3-ol, thus indicating that the substrate competes with molecular oxygen for reducing equivalents from NADH through the reductase. (Note that the endogenous oxidation of NADH by the reductase is a common observation with flavoproteins and results in the formation of oxygen reduction products.)

Possible Involvement of 3-octanol as an Intermediate in the Conversion of 1-octen-3-ol to 3-octanone

When using partially purified preparations of reductase, NADH addition was an absolute requirement for the conversion of 1-octen-3-ol to 3-octanone. Despite the requirement of this reaction for NADH, no net oxidation of NADH occurs, as shown in Table 7.

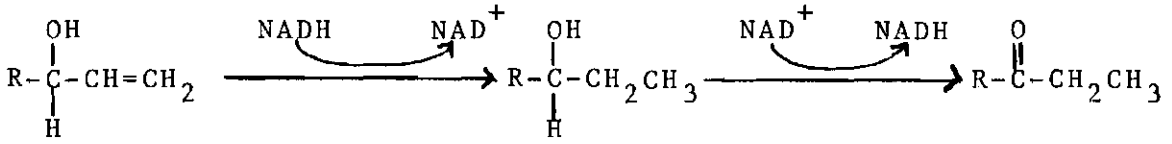
Table 7. Spectrophotometric Determination of NADH Oxidation During the Reductase Catalyzed Conversion of 1-octen-3-ol to 3-octanone

System	NADH Oxidation Rate[1]	
	Anaerobic	Aerobic
Reductase, NADH	0.00	0.015
Reductase, NADH, 1-octen-3-ol	0.00	0.015

[1]Absorbance change at 340 nm in 120 seconds.

In an air saturated solution consisting of only reductase and NADH, the usual small rate of NADH oxidation occurs corresponding to the reductase mediated transfer of electrons from NADH to molecular oxygen (endogenous). Upon addition of 1-octen-3-ol to this system, no net change in the rate of NADH oxidation is observed. When these reactions are carried out under anaerobic conditions, endogenous oxidation of NADH in the presence of just reductase is not observed, as expected. It is evident that upon addition of the substrate 1-octen-3-ol, NADH oxidation is still not observed. In both the aerobic and anaerobic experiments, g.c. analysis confirmed that formation of the product 3-octanone was occurring. These results can only

be reconciled with the absolute requirement for NADH if in the conversion of 1-octen-3-ol to 3-octanone, NADH is being oxidized and then reformed in a two step process. In addition, the data in Table 5 show that a small amount of 3-octanol (300 µg/ml) is also detected during the reaction of 1-octen-3-ol. Taken together, these results suggest the involvement of 3-octanol as an intermediate as illustrated below.



The fact that NAD^+ cannot substitute for NADH in the reductase catalyzed ketonization of 1-octen-3-ol indicates that this reaction does not proceed through the α, β-unsaturated ketone, i.e., 1-octen-3-one. In line with this, 1-octen-3-one was converted to 3-octanone by cell-free preparations with product yields of only 200 µg/ml whereas the cell free incubation of 3-octanol resulted in 3-octanone yields of 1200 µg/ml (Table 5). Since the pathway proposed above involves initial oxidation of NADH concommittant with the reduction of a carbon-carbon double bond followed by reduction of the newly formed NAD^+ during ketone formation, we predict

that incubation of 3-octanol with reductase and NAD^+ should result in both NADH formation and ketone formation. This, in fact, is the case as shown in Table 8.

Table 8. Spectrophotometric Determination of NADH Formation During Reductase Catalyzed Conversion of 3-octanol to 3-octanone

System	NADH Formation	
	Anaerobic	Aerobic
Reductase, NAD^+	0.00	0.00
Reductase, NAD^+,	0.002[2]	N.D.[1]
3-octanol	0.020[3]	N.D.[1]

[1]Could not be determined because of endogenous oxidation of NADH.

[2]Absorbance increase at 340 nm in 120 sec.

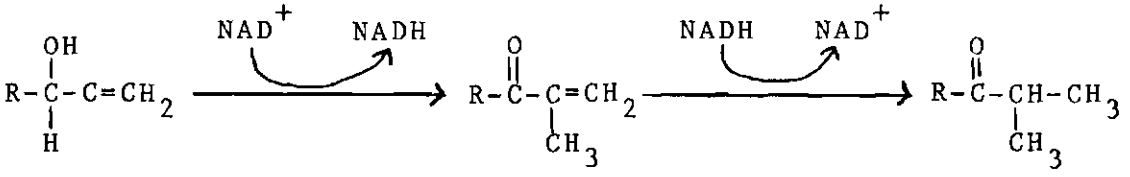
[3]Absorbance increase at 340 nm in 1200 sec.

NADH formation could not be detected in a solution consisting of reductase, NAD^+ and 3-octanol under aerobic conditions, presumably because once formed NADH is reoxidized. However, under anaerobic conditions, the endogenous oxidation of NADH is eliminated and thus NADH formation is observed. Strong support for the proposed intermediacy of 3-octanol is

provided by the data in Table 5, which establish that 3-octanone is formed from this substrate in amounts which can easily account for the product formed from 1-octen-3-ol.

Possible Involvement of 2-methyl-1-hepten-3-one as an Intermediate in the Conversion of 2-methyl-1-hepten-3-ol to its' Saturated Ketone

In contrast to the results obtained with 1-octen-3-ol, the minor product detected from the cell-free incubation of 2-methyl-1-hepten-3-ol is apparently the α,β-unsaturated ketone. As shown in Table 5, the incubation of the allylic alcohol and the unsaturated ketone using cell-free preparations both gave comparable yields of the saturated ketone. Therefore, this reaction appears to proceed through the α,β-unsaturated ketone as illustrated below.



In the presence of partially purified reductase and NAD^+, 2-methyl-1-hepten-3-ol was converted to both 2-methyl-1-hepten-3-one and 2-methyl-3-heptanone with yields 200 µg/ml and 250 µg/ml respectively. In addition, the incubation of the unsaturated ketone with reductase and NADH results in the formation of the saturated ketone.

CHAPTER IV

DISCUSSION

Because of the unusual specificity and stereoselectivity of the P. oleovorans epoxidation system and the fact that large quantities of the enzymatic products can be generated, this system provides an excellent choice for mechanistic studies on oxygen activation. In addition, the enzymatic epoxidation reaction finds a chemical analogy in peracid epoxidations which are perhaps the most well studied and best understood examples of "oxenoid" reactions in organic chemistry. Although the "oxenoid" mechanism has been widely proposed in accounting for many monooxygenase reactions, evidence for such a mechanism has been only indirect. Perhaps the strongest evidence of an "oxenoid" mechanism is the occurrence of the NIH shift during aromatic hydroxylations. The NIH shift indicates the formation of an arene oxide; however, it remains to be established how the arene oxide is generated. Since a diagnostic characteristic of an "oxenoid" mechanism is the syn addition of oxygen, we were able to directly test for this mechanism in the P. oleovorans system by determining whether or not the epoxidation reaction involves retention of the original olefinic geometry in the epoxide product. Because of the

specificity of this enzyme system, internal olefins could not be used in such a study, and we therefore carried out the synthesis of trans,trans-1,8-dideuterio-1,7-octadiene. This deuterated diene provided an ideal substrate for our studies. Because of the absence of a terminal methyl group, there were no side products that could have resulted from the hydroxylation reaction. Furthermore, the substrate provided an additional carbon-carbon double bond which we could examine for possible olefin isomerization processes during the course of the epoxidation reaction. Although only the trans isomer of the deuterated olefin was synthesized, there was however, the formation of significant amounts of fully protonated 1,7-octadiene. The enzymatic epoxidation of this preparation therefore gave rise to products which were mixtures of both deuterated and fully protonated epoxides. As a result, the proton NMR spectrum of the product mixture exhibited serious overlapping in the spectral regions of interest and thus it was difficult to make spectral assignments and to quantitatively analyze the mixture. In order to overcome this difficulty, we used partially relaxed proton Fourier Transform NMR. This NMR procedure, which provides for the resolution of overlapping NMR signals of nuclei with different relaxation times,[76,77] was applicable to our studies since the epoxide and olefin protons in corresponding positions of deuterated and nondeuterated molecules would have substantially different relaxation

rates. Because of the nature of this technique, it was a real advantage that the product mixtures contained both deuterated and fully protonated species. This allowed us to confirm spectral assignments on the basis of a comparison of the relaxation rates of the deuterated and protonated species. In addition, we were able to look for secondary deuterium isotope effects by comparing the relative deuterium content of the epoxide and olefin portions of the 7,8-epoxy-1-octene product.

A quantitative determination of the epoxide region of the enzymatic product using relaxation analysis revealed that the product contained 70% cis and 30% trans deuterated epoxide and a determination of the olefin region of the product revealed the presence of only the trans isomer. The fact that only the trans olefin was present not only substantiated that the starting material was of very high configurational purity but also indicated that no isomerization of the double bond occurred during the course of the reaction. With regard to the epoxide region, what the results of these studies establish is that the configuration about the double bond in a simple olefin is not maintained in the epoxide product isolated after reaction with the P. oleovorans system. Simple mechanisms involving the concerted addition of an electrophilic activated oxygen species do not predict such a result. Thus, these results are consistent with the conclusion that a straightforward

"oxenoid" mechanism, in the usual sense of the term (i.e., comparable to that operative in peracid, and possibly in transition metal oxo complex, epoxidations) is not operative in this system, although it is certainly conceivable that acceptable complex variations of such a mechanism (e.g., those involving generation of transitory intermediates with altered configurations "at the active site") could be formulated.[82] In line with this conclusion, May and coworkers have recently noted that the chemical reactivity patterns observed with the P. oleovorans system are far different than those observed in peracid epoxidations.[23]

It should be kept in mind that our results were obtained with resting cells, and the possible complications inherent in mechanistic analysis of such data must be considered. For example, it might be postulated that some of the isolated epoxide product is generated from octadiene by enzymes other than the "epoxidation/hydroxylation" system and these other enzymes may operate via a different mechanism. In several years of detailed reactivity, specificity and stereochemical studies using whole cells, cell-free systems and purified enzymes, we have obtained no evidence for such mechanistic diversity. However, even if this hypothetical situation were to exist in P. oleovorans, the basic conclusion that a simple concerted mechanism of oxygen addition does not predict the major (cis) product we obtain would still hold,

since isomerization "after" product release is eliminated by our control experiments. Thus, for the purposes of mechanistic analysis, the situations were either a single enzyme system or several enzyme systems with similar reactivity characteristics are operative. These systems represent identical models, and we have no basis on which to distinguish them.

A second conceivable complication in this system involves the possible existence of isomerization processes which might not have been eliminated by the control experiments. For example, a specific "isomerase" could be present which functions in concert with the "epoxidase", and isomerizes only newly formed epoxides before their release into free solution. Although one cannot eliminate this possibility with certainty, we know of no precedent of such a situation, and it seems unlikely that such an "isomerase" would account for the net "inversion" of configuration. Furthermore, the stereochemical results demonstrate that the epoxidation of 1,7-octadiene gives optically active epoxides with a high degree of optical purity and also that the stereochemical configuration of a preformed epoxide functionality does not undergo significant isomerization during diepoxidation by whole cells. These results are not easily reconciled with the existence of such an "isomerase".

With these considerations in mind, it is possible

to make tentative evaluations of other mechanistic proposals by considering the lack of configurational retention together with the highly directional attack of oxygen at the incipient asymmetric carbon (C-2) atom required by our stereochemical results. For example, a two step mechanism in which the activated oxygen species attacks at one of the olefinic carbon atoms from a preferred direction to generate a carbonium ion or radical intermediate, which subsequently closes to the epoxide product might be suggested. Indeed such species as "$HO^{\cdot}$" or "HO^{+}", have often been considered as possible candidates for activated oxygen in oxygenase reactions. Mechanisms involving analogous intermediates can be envisioned for the reactions involving superoxide or triplet oxygen atom, both of which have been considered as being potentially involved in reactions of oxygenases or model systems. (For the purposes of this discussion, the designation "HO^{+}", should be considered a formalism; it cannot be considered a real mechanistic possibility which has a chemical analogy.)

With a two step mechanism, four possible variants exist and these possibilities are illustrated in Figures 18 and 19. Figure 18 illustrates two possible variants of a two step mechanism in which the activated oxygen species attacks at C-2. Prior attack at C-2 from the <u>si</u>-<u>re</u> face[78] to generate a carbonium ion or radical intermediate followed by closure before and after bond rotation, would give rise

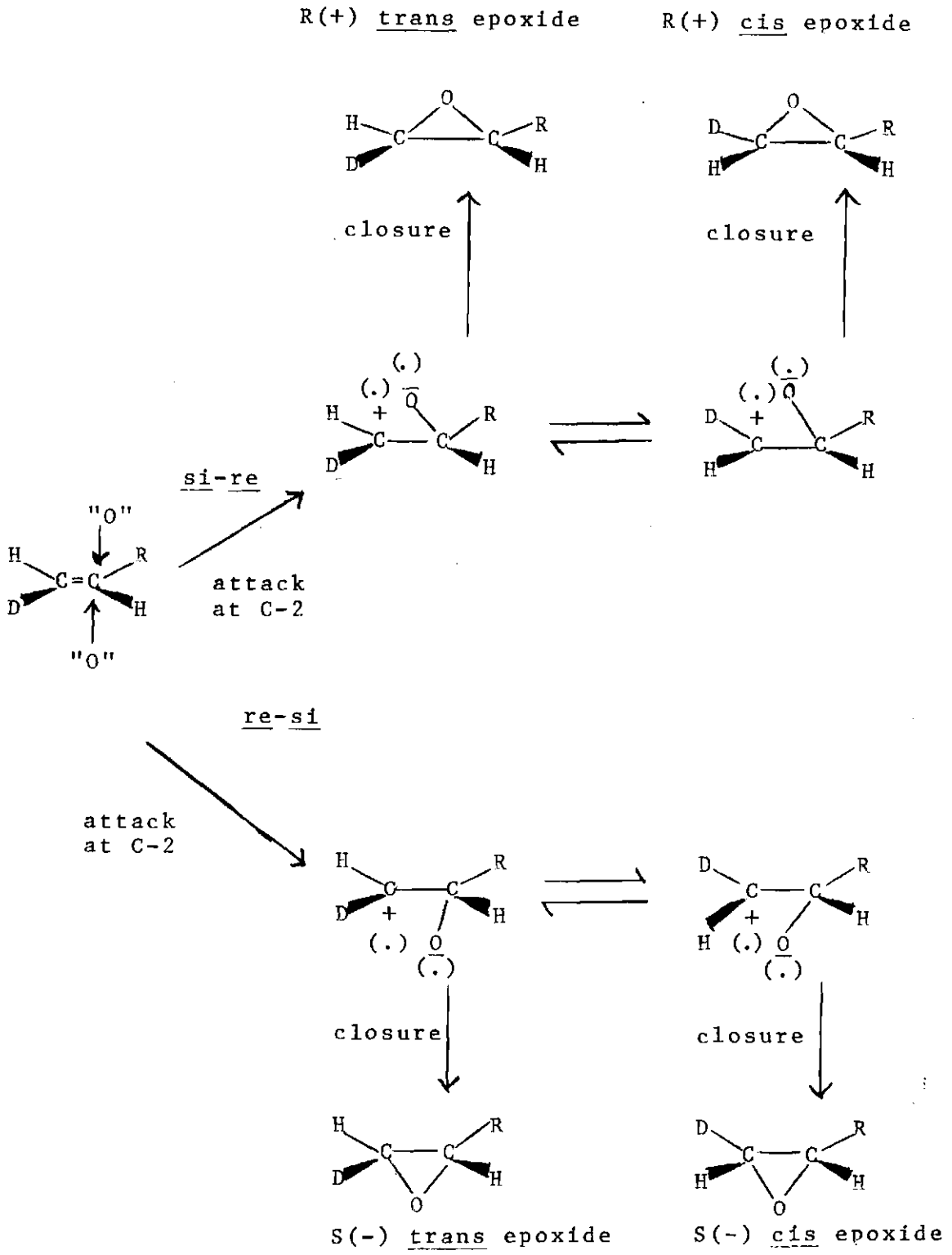


Figure 18. Two Variants of a Two Step Mechanism Involving Prior Attack at C-2

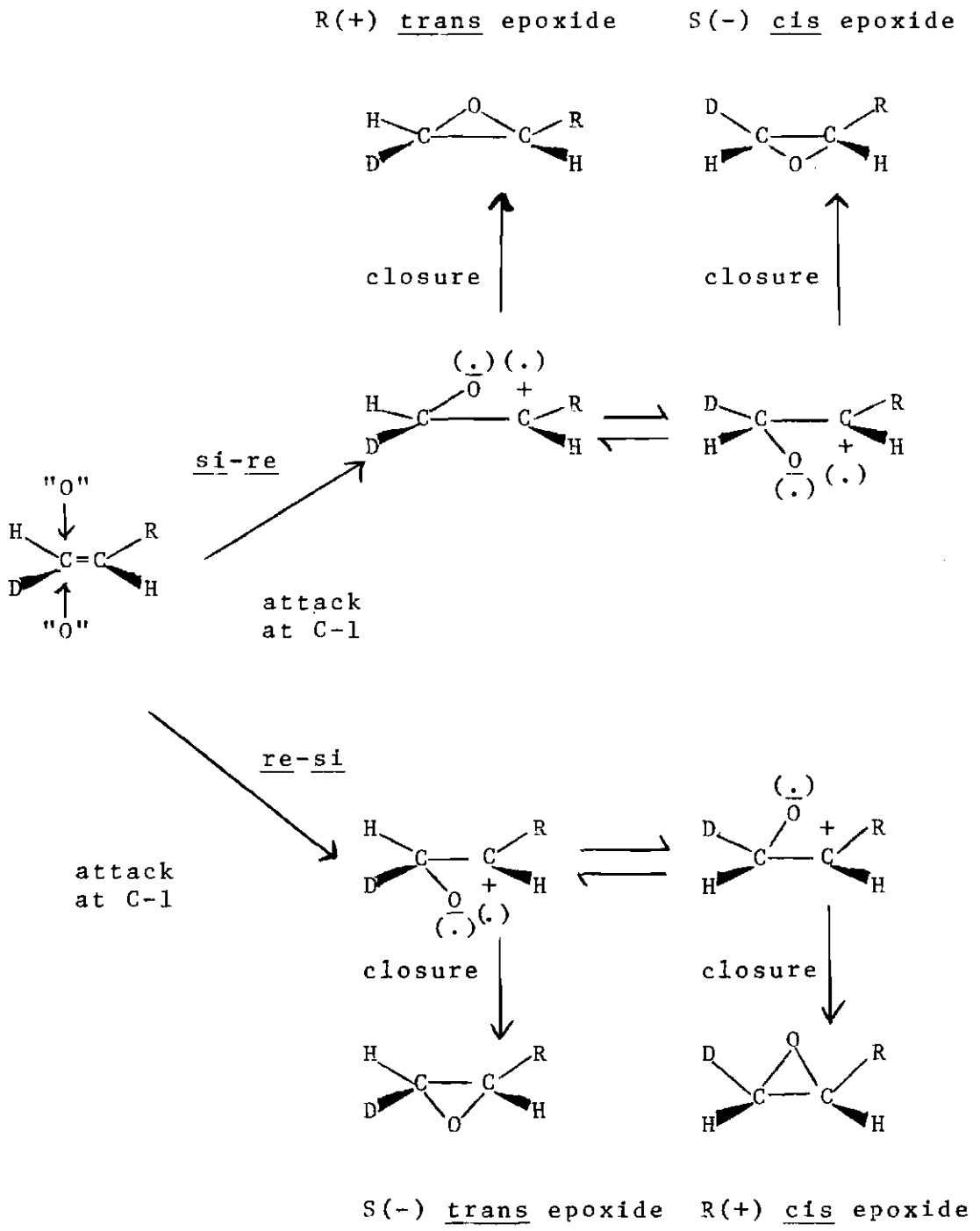


Figure 19. Two Variants of a Two Step Mechanism Involving Prior Attack at C-1

to both cis and trans epoxide of the R(+) configuration. However, such a result is not fully consistent with our results since it would allow, at most, formation of 50% cis epoxide and 50% trans epoxide and not the observed results of 70% and 30%, respectively. On the other hand, prior attack at C-2 from the re-si face of the double bond cannot account for the observed stereochemistry at C-2 since closure to the epoxide before or after bond rotation would produce epoxides of only the S(-) configuration.

As shown in Figure 19, the other two possible variants of a two step mechanism would involve prior attack at C-1. Prior attack at C-1 from the re-si face to generate an analogous intermediate is inconsistent with our results since only the cis epoxide generated by closure after carbon-carbon bond rotation-and not the trans epoxide generated by syn closure-would have the R(+) configuration. Similarly, prior attack at C-1 from the si-re face would generate only the trans epoxide having the R(+) configuration and thus is inconsistent with our results. Therefore, of the four possible variants of a two step mechanism, none is fully consistent with our results.

Another interesting possibility that can be suggested is the initial formation of a cis 1,2-diol intermediate as shown in Figure 20. If the formation of a cis 1,2-diol occurs at the si-re face, then syn closure to the epoxide via attack at C-1 or C-2 would give the trans epoxide with

the R(+) configuration. If closure to the epoxide occurs after bond rotation via attack at C-1, thus displacing the terminal hydroxyl group, then the product formed would be the cis epoxide with the R(+) configuration. The predominance of the cis deuterated epoxide product might reflect the preference of this process for a trans orientation of the attacking and leaving hydroxyl groups, which is attained only after bond rotation. Such a pathway would be consistent with our results.

On the other hand, formation of a cis 1,2-diol at the re-si face of the double bond would not be consistent with our results since neither a syn closure nor closure after bond rotation via C-1 or C-2 attack can provide for the trans epoxide having the R(+) configuration, and this represents 30% o- the product. Of course, combinations of these various pathways or a combination with a two step process can also be envisioned, and the data at hand are insufficient to distinguish between them.

Although formation of a cis 1,2-diol at the si-re face would be consistent, we have obtained no evidence for the formation of such intermediates but it is conceivable that such a species may be formed and remain bound at the active site. It is interesting to note that a cis 1,2-diol has been shown to be formed from napthalene by the oxygenase system of P. putida,[79] and that epoxides have been identified as intermediates in the conversion of simple aliphatic

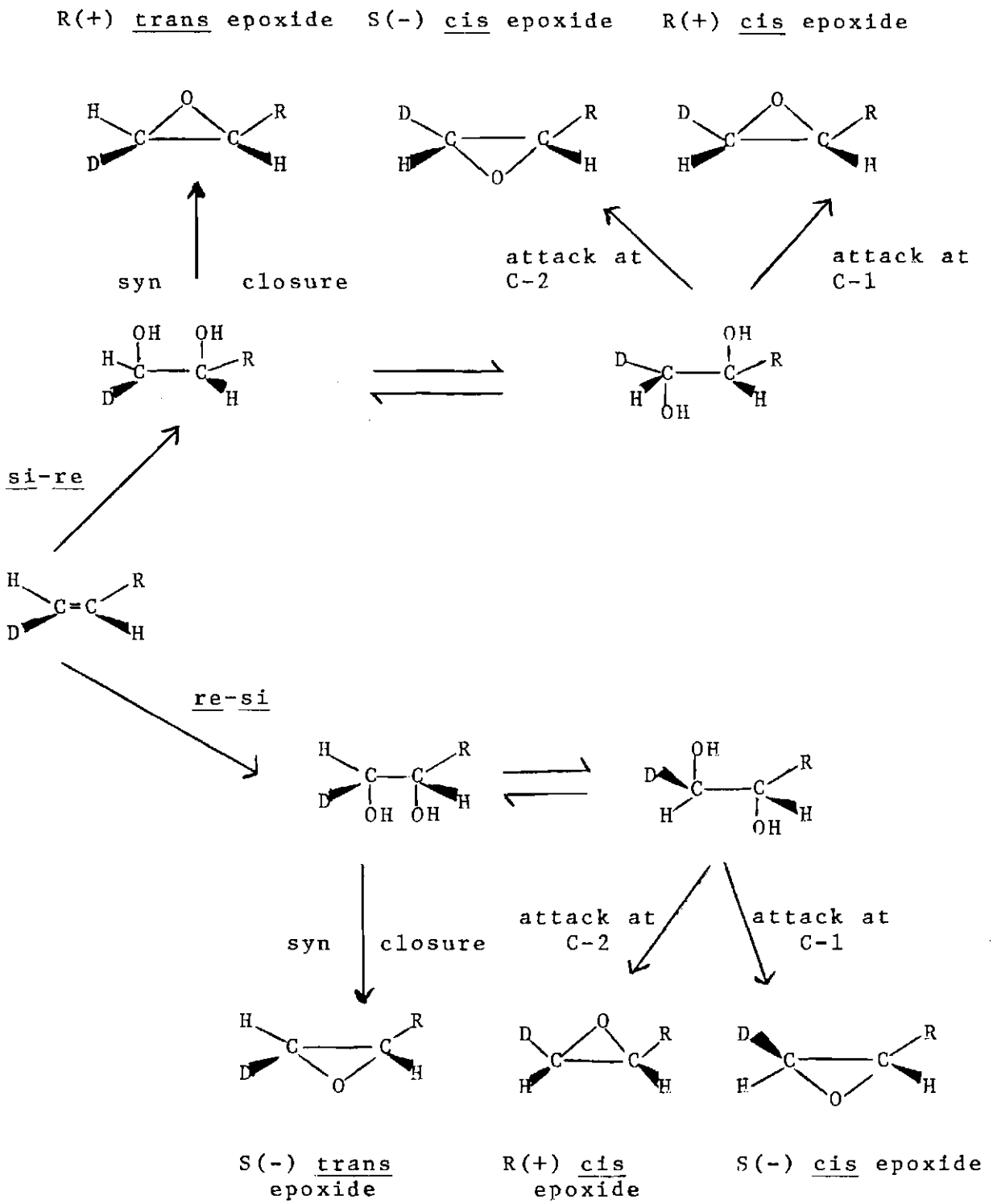


Figure 20. Mechanistic Schemes Involving Initial Formation of a cis 1,2-diol

olefins to glycols by liver microsomes.[80]

Although it is evident that the detailed pathway of this enzymatic oxygenation reaction has not yet been fully defined, the results presented have provided information which must be taken into account when formulating mechanistic proposals. The question of whether the mechanism of oxygen insertion operative in this system is an unusual one or is in fact, representative of other mono-oxygenase systems as well, must await further experimental data. However, it should be kept in mind that the P. oleovorans system differs from the hydroxylation system of P. putida, liver microsomes, and adrenocortical mitochondria in that it does not contain cytochrome P-450.

Concerning the nature of the activated oxygen species, it is difficult to make reasonable suggestions for possible structures and characteristics of the actual enzymic reagent based on the results at hand. Therefore, it would be of considerable interest to find a model system which mimics the features of the P. oleovorans system. The Udenfriend system, which has been considered as a possible model for oxygenases,[49] could be examined with the deuterated olefin used in our studies. This model system apparently does not operate by an "oxenoid" mechanism since a significant NIH shift is not observed during hydroxylation of aromatic compounds. Other chemical systems, such as the base catalyzed hydrogen peroxide mediated epoxidations using quaternary

salts derived from alkaloids,[81] could also be investigated. Although the enantioselectivity of these reactions are low, they have recently been shown to involve the formation of optically active epoxides from electron-poor olefins.

Since our results clearly demonstrate that an "oxenoid" mechanism is not operative in this enzymatic system, it might be suggested that the activated oxygen species is not electron difficient. This could be tested by studying the effects of electron-withdrawing and electron-donating groups on the epoxidation reaction. Alternatively, if such substitution poses a steric problem, an investigation of the reactivity of conjugated dienes towards epoxidation could be carried out. For example, 1,3,7-octatriene would serve as an ideal substrate for this study. The conjugation of the terminal double bond would result in a delocalization of pi electrons thus reducing the electron density of the double bond. If the activated oxygen species is electrophilic, epoxidation might occur at the nonconjugated double bond only.

Regardless of this and any other mechanistic considerations, it is important to note that the unusual selectivity towards terminal double bonds, the lack of configurational retention and the fact that the conversion of 1,7-octadiene to 7,8-epoxy-1-octene proceeds with a high degree of stereoselectivity, each imparts considerable synthetic potential to this system. These characteristics

cannot be duplicated by any known chemical epoxidizing agent.

In addition to the high stereoselective synthesis of monoepoxides, our results also demonstrate that essentially the same degree of stereoselectivity occurs in the diepoxidation of simple olefins by this enzyme system. We examined the epoxidation of racemic 7,8-epoxy-1-octene to determine what effect, if any, the stereochemistry at one end of the molecule has upon the direction of attack at the other end. The results presented establish that the diepoxide product produced from racemic monoepoxide exhibits an enantiomeric excess of R sites of only 20% while the diepoxide produced from octadiene, through the monoepoxide intermediate which is generated almost exclusively with the R configuration, exhibits an enantiomeric excess of R sites of nearly 70%. Thus, it is clear that the opposite ends of a straight chain substrate do not operate independently, but that the configuration of a preformed epoxide functionality indeed influences the stereochemical course of oxygen insertion in the diepoxidation reaction.

These findings are important in several respects. They establish the unique suitability of this system for the facile production of both mono- and diepoxides of high optical purity. They establish a basis for control of the stereochemistry of this enzymatic reaction in order to produce epoxides of desired configuration and they provide

valuable insight into the nature of substrate binding and the topography of the active site region in this system. In addition, the fact that the unreacted monoepoxide during the diepoxidation of racemic 7,8-epoxy-1-octene exhibits an excess of S(-) sites, confirms our contention that once formed, the epoxide functionality does not isomerize to any significant extent.

Perhaps the most attractive feature of this enzyme system for practical synthetic applications is the fact that optically active epoxides can be produced on a preparative scale. The epoxidation reactions carried out with growing cultures, resting cell suspensions and cell-free extracts are simple procedures in which the products can be obtained in gram quantities. Each method, however, has its limitations in that percent conversions at best approach 20-25%. Undoubtedly, such factors as product toxicity, product inhibition and product metabolism play major roles in accounting for the limited conversions.

Our preliminary work with the incorporation of an organic solvent into a resting cell suspension and especially the work of Schwartz and McCoy[67] with mixed solvent fermentations indicate the potential that exists in optimizing the efficiency of this enzyme system. This procedure takes advantage of the insolubility of the epoxide products in an aqueous medium and thus allows for higher product yields by minimizing produce exposure and

the subsequent toxic effects to the cells in the aqueous phase.

It would be more desirable; however, to have in hand optimized conditions for producing cell-free epoxidizing preparations since they are easier to handle in most cases than whole cells. An optimization would greatly enhance the attractiveness of this enzyme system and thus its utilization for synthetic purposes.

With regard to possible product metabolism, it is apparent that further oxidation of the product 7,8-epoxy-1-octene to give the diepoxide cannot alone account for the decrease in monoepoxide concentration which occurs with prolonged incubation using whole cells or cell-free extracts. Huybregste and Van der Linden have provided evidence that octane grown cells of P. aeruginosa can metabolize 1,2-epoxyoctane to give initially a 1,2-diol followed by oxidation to form α-hydroxyoctanoic acid.[26] In contrast, Abbott and Hou suggest that 1,2-epoxyoctane is metabolized by P. oleovorans by an initial hydroxylation of the terminal methyl group.[58] In view of the fact that allylic alcohols are converted to saturated ketones by P. oleovorans, the metabolism of 7,8-epoxy-1-octene might proceed initially by reduction of the carbon-carbon double bond and subsequent oxidation of the newly formed methyl group. Although each of the above possibilities might be suggested to account for epoxide loss, we have not identified

any degradation products to support or refute either pathway.

In an attempt to further explore the stereochemical aspects of the epoxidation reaction, we investigated several allylic alcohols as possible substrates for the P. oleovorans system. We were particularly interested in determining whether the stereoselectivity observed in the epoxidation of simple, unsubstituted olefins is affected by the presence of an allylic alcohol functionality. The ability of the P. oleovorans system to directly catalyze the conversion of allylic alcohols to their corresponding saturated ketones was one of the most unexpected findings from our studies. The results presented establish that in addition to being centrally involved in the epoxidation/hydroxylation system as an electron-transferring protein, the reductase component in the presence of NADH also converts 1-octen-3-ol to 3-octanone and 2-methyl-1-hepten-3-ol to 2-methyl-3-heptanone. These reactions do not involve the coupling of additional proteins, do not require molecular oxygen and are not catalyzed by the prosthetic group alone or by other flavin-containing proteins, thus indicating a new and unusual feature of the reductase component. The fact that ketonization is catalyzed by the reductase component alone suggests an active site different from that of the epoxidation and/or hydroxylation reactions. In addition, despite the presence of a terminal methyl group

and a terminal double bond, neither hydroxylation nor epoxidation occurs to any appreciable degree when the allylic alcohols were incubated with whole cells or cell-free extracts. It has previously been noted that in the epoxidation/hydroxylation system, the methylene groups of a straight chain hydrocarbon are critical for the proper binding of these compounds within the active site.[22] Presumably, this active site is of a hydrophobic nature. If the carbon-carbon double bond region of the olefin is most critical for proper binding, then the hydrophilic hydroxyl substituent may prevent this proper binding required for epoxidation. Alternatively, the hydroxyl group through steric interactions may prevent access to the active site region. The results at hand cannot differentiate between these two possibilities. However, a better understanding concerning this question may be obtained by investigating the reactivity of hydrophobic substituted olefins, (e.g., 3-methyl-1-octene) towards epoxidation.

Of the various allylic alcohols investigated, only linalool (3,7-dimethyl-1,6-octadien-3-ol) was nonreactive. Linalool differs from the other substrates in that it is a tertiary alcohol and not a secondary alcohol. Ketonization of the secondary alcohols requires the removal of hydrogen at C-3 and the nonreactivity of linalool may reflect the inability of the enzyme to remove the methyl group at C-3. In view of the apparent involvement of 3-octanol as an

intermediate in the conversion of 1-octen-3-ol to 3-octanone, a mechanism involving a reductase-NAD^+ mediated intramolecular or intermolecular migration of a hydride from C-3 to C-1 would not be consistent with the data at hand. However, such a mechanism could be operative in the conversion of 2-methyl-1-hepten-3-ol to its saturated ketone via the α,β-unsaturated ketone intermediate as illustrated in Figure 21.

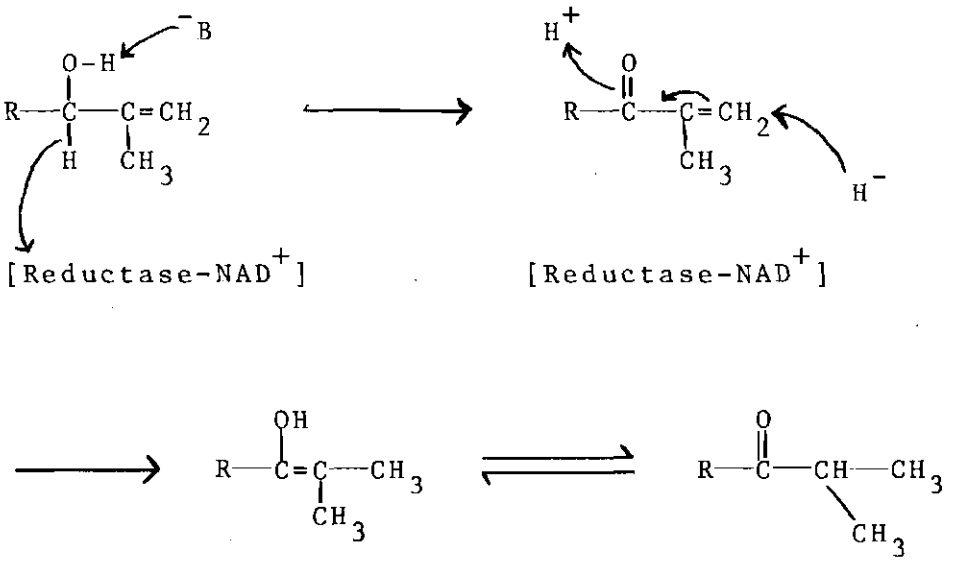


Figure 21. Mechanism Involving a Reductase-NAD^+ Mediated Intramolecular Hydride Migration

To test for this possible pathway, one could investigate a labeled allylic alcohol as a substrate in which deuterium is substituted for hydrogen at C-3. A NMR or mass spectral analysis would reveal whether the label is migrated to C-1.

Although it is clear that additional experimental data is required for a better understanding of the mechanism of these reactions, the above suggestion can be regarded as a working hypothesis for future studies.

REFERENCES AND NOTES

1. Hayaishi, O., Katagiri, M., and Rothberg, S., J. Am. Chem. Soc., 77, 5450, (1955).

2. Mason, H. s., Fowlks, W. L., and Peterson, E., J. Am. Chem. Soc., 77, 2914 (1955).

3. Hayaishi, O., "Molecular Mechanisms of Oxygen Activation," O. Hayaishi, Ed., Academic Press, New York, N.Y., Chapter 1.

4. Peterson, J. A., Basu, D., and Coon, M. J., J. Biol. Chem., 241, 5162 (1966).

5. Peterson, J. A., Kusnose, M., Kusnose, E., and Coon, M. J., J. Biol. Chem., 242, 4334 (1967).

6. Peterson, J. A., and Coon, M. J., J. Biol. Chem., 243, 329 (1968).

7. McKenna, E. J., and Coon, M. J., J. Biol. Chem., 245, 3882 (1970).

8. Lode, E. T., and Coon, M. J., J. Biol. Chem., 246, 791 (1971).

9. Ueda, T., Lode, E. T., and Coon, M. J., J. Biol. Chem., 247, 2109 (1972).

10. Benson, A., Tomoda, K., Change, J., Matsueda, G., Lode, E. T., Coon, M. J., and Yasunobu, K. T., Biochem. Biophys. Res. Comm., 42, 640 (1971).

11. Ueda, T., and Coon, M. J., J. Biol. Chem., 247, 5010 (1972).

12. Ruettinger, R. T., Olson, S. T., Boyer, R. F., and Coon, M. J., Biochem. Biophys. Res. Comm., 57, 1011 (1974).

13. Katagiri, M., Ganguli, B. N., and Gunsalus, I. C., J. Biol. Chem., 243, 3543 (1968).

14. Gunsalus, I. C., Hoope-Seyler's Z. Physiol. Chem., 349, 1610 (1968).

15. Tyson, C. A., Lipscomb, J. D., and Gunsalus, I. C., J. Biol. Chem., 247, 5777 (1972).

16. Cardini, G., and Jurtshuk, P., J. Biol. Chem., 243, 6070 (1968).

17. Omura, T., Sanders, E., Eastabrook, R. W., Cooper, D. Y., and Rosenthal, O., Arch. Biochem. Biophys., 117, 660 (1966).

18. Scheyer, H., Cooper, D. Y., and Rosenthal, O., J. Biol. Chem., 246, 6103 (1972).

19. Lee, A. Y. H., and Coon, M. J., J. Biol. Chem., 243, 1331 (1968).

20. Lu, A. X. H., Levin, W., West, S. B., Jacobson, J., Ryan, D., Kuntzman, R., and Cooney, A. H., J. Biol. Chem., 248, 456 (1973).

21. May, S. W., and Abbott, B. J., Biochem. Biophys. Res. Comm., 48, 1240 (1972).

22. May, S. W., and Abbott, B. J., J. Biol. Chem., 248, 1725 (1973).

23. May, S. W., Schwartz, R. D., Abbott, B. J., and Zaborsky, O. R., Biochim. Biophys. Acta, 403, 245 (1975).

24. May, S. W., and Schwartz, R. D., J. Am. Chem. Soc., 96, 4031 (1974).

25. May, S. W., and Abbott, B. J., Abstracts, 166th National Meeting of the American Chemical Society, Chicago, Ill., Aug 1973, Biol. No. 218.

26. May, S. W., Abbott, B. J., and Felix, A., Biochem. Biophys. Res. Commun., 54, 1540 (1973).

27. May, S. W., Abbott, B. J., and Schwartz, R. D., Prep., Div. Pet. Chem., Am. Chem. Soc., 19, 713 (1974).

28. May, S. W., "Catalysis in Organic Synthesis," Academic Press, New York, N.Y., 1976, pp. 101-111.

29. May, S. W., Steltenkamp, M. S., Schwartz, R. D., and McCoy, C. J., J. Am. Chem. Soc., 98, 7856 (1976).

30. May, S. W., Gordon, S. L., and Steltenkamp, M. S., J. Am. Chem. Soc., 99, 2017 (1977).

31. Van der Linden, A. C., Biochim. Biophys. Acta, 77, 157 (1963).

32. Huybregste, R., and Van der Linden, A. C., Antonie van Leeuwenhoek, 30, 185 (1964).

33. Hamilton, G. A., "Molecular Mechanisms of Oxygen Activation", O. Hayaishi, Ed., Academic Press, New York, N.Y., Chapter 10.

34. Hamilton, G. A., J. Am. Chem. Soc., 96, 3391 (1964).

35. Hamilton, G. A., Adv. Enzymol. Relat. Subj. Biochem., 32, 55 (1969).

36. Hamilton, G. A., Prog. Bioorg. Chem., 1, 83 (1971).

37. Hamilton, G. A., Ann. N.Y. Acad. Sci., 212, 4 (1973).

38. Swern, D., J. Am. Chem. Soc., 69, 1692 (1947).

39. Swern, D., Org. React., 7, 378 (1953).

40. Berti, G., Top. Stereochem., 7, 93 (1973).

41. House, H. O., "Modern Synthetic Reactions", 2nd ed., W. A. Benjamin, Menlo Park, Calif., pp. 296-307 (1972).

42. Witnauer, L. P., and Swern, D., J. Am. Chem. Soc., 72, 336 (1950).

43. Lynch, D. M., and Pausacker, K. H., J. Am. Chem. Soc., 77, 1525 (1955).

44. Sharpless, K. B., Flood, T. C., J. Am. Chem. Soc., 93, 2316 (1971).

45. Korse, W., Chem. Commun., 1610 (1968).

46. Sharpless, K. B., Townsend, J. M., and Williams, D. R., J. Am. Chem. Soc., 94, 295 (1972).

47. Hiatt, R., "Oxidation", Vol. 2, R. L. Augustine, Ed., Marcel Dekker, New York, N.Y., (1971), Chapter 3.

48. Jerina, D. M., Chem. Technol., 4, 120 (1972).

49. Udenfriend, S., Clark, C. T., Axelrod, J., and Brodie, B. B., J. Biol. Chem., 208, 731 (1954).

50. Ullrich, V., Z. Naturforsch, 246, 699 (1969).

51. Frommer, U., and Ullrich, V., Z. Naturforsch., 260, 322 (1971).

52. Hamilton, J. A., and Friedman, J. P., J. Am. Chem. Soc., 85, 1008 (1963).

53. Mager, H. J. X., and Berends, W., Biochim. Biophys. Acta., 118, 440 (1966).

54. Bowers, A., Halsall, T. G., Jones, E. R. H., and Lemin, A. J., J. Chem. Soc., 2555 (1953).

55. Brown, H. C., and Gupta, S. K., J. Am. Chem. Soc., 93, 1816 (1971).

56. Newson, H., and Woods, W. G., Inorg. Chem., 7, 177 (1968).

57. Vold, R. L., Waugh, J. S., Klein, M. P., and Phelps, D. L., J. Chem. Phys., 48, 3831 (1968).

58. Abbott, B. J., and Hou, C. T., Appl. Microbiol., 26, 86 (1973).

59. Schwartz, R. D., and McCoy, C. J., Appl. Microbiol., 26, 217 (1973).

60. Schwartz, R. D., and McCoy, C. J., Appl. Microbiol., 31, 78 (1976).

61. Schwartz, R. D., and McCoy, C. J., Appl. Microbiol., 34, 47 (1977).

62. Strobel, H. W., Lu, A. Y. H., Heidema, J., and Coon, M. J., J. Biol. Chem., 245, 4851 (1970).

63. The terms fully protonated and cis or trans deuterated are used in this paper with reference to a given epoxide or olefin functionality, without regard to the deuterium content of functionalities at the other end of the molecule. The NMR, of course, looks only at the aggregate of epoxide or olefin sites.

64. Pople, J. A., Schneider, W. G., and Bernstein, H. J., "High Resolution Nuclear Magnetic Resonance", McGraw-Hill, New York, N.Y., 1959.

65. The standard NMR spectral notation of reference 64 is used with increasing alphabetical order indicating increasing field.

66. Reilly, C. A., and Swalen, J. D., J. Chem. Phys., 32. 1378 (1960).

67. Williamson, K. L., Sanford, C. A., and Nicholson, C. R., J. Am. Chem. Soc., 86, 762 (1964).

68. Miller, C. R., and Gordon, S. L., J. Chem. Phys, 53, 3531 (1970).

69. Brown, P. M., Krishna, N. R., and Gordon, S. L., J. Magn. Reson., 20, 540 (1975).

70. Abragam, A., "Principles of Nuclear Magnetism", Oxford University Press, London, 1961, Chapter VIII.

71. Sutton, L. E., Ed., Chem. Soc., Spec. Publ., No. 2 (1958).

72. Coke, J. L., and Shue, R. S., J. Org. Chem., 38, 2210 (1973).

73. See Morrison, J., and Mosher, H., "Asymmetric Organic Reactions", Prentice Hall, Englewood Cliffs, N.J., p. 258-262 (1971).

74. Henbest, H. B., and Wilson, R. A. L., J. Chem. Soc., 1958 (1957).

75. Sharpless, K. B., and Michaelson, R. C., J. Am. Chem. Soc., 95, 6136 (1973).

76. Allerhand, A., Clouse, A. O., Rietz, R. R., Roseberry, T., and Schaffer, R., J. Am. Chem. Soc., 94, 2445 (1972).

77. Patt, S. L., and Sykes, B. D., J. Chem. Phys., 56, 3182 (1972).

78. In this discussion, the Hanson prochirality nomenclature for the trans deuterated substrate is used (see R. Bently, "Molecular Asymmetry in Biology," Vol. I., Academic Press, New York, N.Y., 1969, Chapter 4), since the mechanistic arguments are based on the cis-trans configuration of the product obtained from this molecule. Note, however, that as far as C-2 of the olefin is concerned, the si-si face of fully protonated octadiene is equivalent to the si-re face of the trans deuterio substrate.

79. Jeffrey, A. M., Yeh, H. J. C., Jerina, D. M., Patel, T. R., Davey, J. F., and Gibson, D. T., Biochemistry, 14, 575 (1975).

80. Maynert, E. W., Foreman, R. L., and Wotabe, T., J. Biol. Chem., 245, 5234 (1970).

81. Helder, R., Hummelin, J. C., Laane, R. W. P. M., Wiering, J. S., and Wynberg, H., Tetrahedron Letters, 21, 1831 (1976).

82. We recognize that, in general, complex mechanisms involving any number of transitory intermediates can always be postulated, and we have confined our discussions to a few simple mechanistic possibilities.

VITA

Michael S. Steltenkamp was born in Covington, Kentucky on May 31, 1951 and lived in Oklahoma City, Oklahoma from 1954 to 1969 with his parents and three sisters. Upon graduating from Bishop McGuinness High School in Oklahoma City in 1969, he attended the University of Oklahoma in Norman, where he received his B.S. in Chemistry in 1973. In the Fall of that year, Michael entered Georgia Institute of Technology where he conducted his graduate work under the guidance of Dr. Sheldon W. May. He graduated in 1978, receiving his Ph.D. in Chemistry.